UNIVERSITY OF CALIFORNIA SAN DIEGO

Characterization of Hippo pathway regulation and the physiological implications of its downstream effectors YAP and TAZ

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

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University of California San Diego
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DEDICATION

This dissertation is dedicated to my mom, Cyndy Plouffe (d.2016), who was an inspiration for me and whose love and dedication made this moment possible.

EPIGRAPH

The heavens declare the glory of God,
and the sky above proclaims his handiwork.

Psalm 19:1

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LIST OF ABBREVIATIONS

AJUBA	Ajuba LIM protein
AMOT	Angiomotin
APC	Adenomatous polyposis coli
ARVC	Arrhythmogenic right ventricular cardiomyopathy
AXL	AXL receptor tyrosine kinase
C3	C3 exoenzyme
CAR	Constitutive androstane receptor
CAMTA1	Calmudulin binding transcription activator 1
CCLE	Cancer cell line encyclopedia
CHX	Cycloheximide
CRC	Colorectal cancer
CRISPR	Clustered regularly interspaced short palindromic repeats
CTGF	Connective tissue growth factor
CTNNA1	Alpha-catenin
CYR61	Cysteine-rich angiogenic inducer 61
2-DG	Deoxy-D-glucose
DSC	Desmocollin
DSG	Desmoglein
DSP	Desmoplakin
DSS	Dextran sodium solfate
DVL	Dishevelled
ECM	Extracellular matrix
EHE	Epithelioid hemangioendothelioma
EMT	Epithelial to mesenchymal transition
FBS	Fetal bovine serum
F/IBMX	Forskolin/3-isobutyl-1-methylxanthine
FILIP1L	Filamin A-interacting protein 1-like
FJX1	Four-jointed
FOSB	FBJ murine osteosarcoma viral oncogene homolog B
GPCR	G-protein coupled receptor
GSK3	Glycogen synthase kinase 3
HCC	Hepatocellular carcinoma
HM	Hydrophobic motif
JUP	Plakoglobin
KO	Knockout
KS	Kaposi sarcoma
KSHV	Kaposi sarcoma-associated herpesvirus
LatB	Latrunculin B
LATS1/2	Large tumor suppressor 1/2
LGR5	Leucine rich repeat containing G-protein coupled receptor 5
LPA	Lysophosphatidic acid
MAP4K	Mitogen-activated protein kinase kinase kinase kinase
MM	Multiple myeloma

MMP-9	Matrix metalloproteinase-9
MOB1A/B	Mob1 homolog
MST1/2	Mammalian STE20-like 1/2
NF2	Neurofibromin 2
NFKb	Nuclear factor-kappa B
NSCLC	Non-small cell lung cancer
PAX3	Paired box 3
PDAC	Pancreatic ductal adenocarcinoma
PKA	Protein kinase A
PKD	Polycystic kidney disease
PKD1/2	Polycystic kidney disease 1/2
P/S	Penicillin/streptomycin
PTPN14	Protein tyrosine phosphatase non-receptor 14
RASSF1A	Ras association domain family member 1A
RCC	Renal cell carcinoma
RHOA	Ras homology family member A
S1P	Sphingosine-1-phosphate
SAV1	Salvador 1
SCRA	Sveinsson's chorioretinal atrophy
TAOK	Tao Kinases
TAZ	WW domain containing transcription factor
TBX5	T-box transcription factor 5
TEAD1-4	TEA domain family members 1-4
TFE3	Transcription factor binding to IGHM enhancer 3
TPA	12-O-tetradecanoylphorbol-13-acetate
UM	Uveal melanoma
UTR	Untranslated region
YAP	Yes-associated protein
VGLL4	Vestigial-like family member 4
vGPCR	Viral GPCR

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FIELDS OF STUDY

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ABSTRACT OF THE DISSERTATION

Characterization of Hippo pathway regulation and the physiological implications of its downstream effectors YAP and TAZ

by

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The Hippo pathway and its downstream effectors, transcriptional coactivators YAP and TAZ, are important for regulating tissue homeostasis and are frequently dysregulated in human disease and cancer. However, it is not clear how the Hippo pathway becomes dysregulated because few mutations have been identified in Hippo pathway components. Therefore, much work in the field has focused on identifying

novel upstream regulators of the Hippo pathway. Nevertheless, it is not always clear which of these components are most physiologically relevant in regulating YAP/TAZ. Thus, to provide a more comprehensive overview of Hippo pathway regulation, we used CRISPR/Cas9 to create knockout cell lines for many of these components and tested their responses to a variety of physiological signals to determine which components are most critical in regulating YAP/TAZ. By this approach, we demonstrate that LATS1/2 are the primary kinases for YAP/TAZ, NF2 and RHOA are important regulators of YAP/TAZ, and TAOK1/3 are direct kinases for LATS1/2.

Moreover, although YAP and TAZ have traditionally been viewed as being more or less redundant, there are evolutionary, structural, and physiological differences that suggest there may be differences in how they are regulated or in their downstream functions. To better characterize the physiological consequences of dysregulated YAP/TAZ, as well as any potential differences between the two, we compared LATS1/2 KO cells, in which YAP/TAZ are constitutively-active, YAP KO, TAZ KO, and YAP/TAZ KO cells. Inactivation of YAP had a strong negative effect on cell spreading, cell volume, glucose uptake, cell proliferation, and migration, while YAP activation in the LATS1/2 KO cells had the opposite effect. Inactivation of YAP had a greater effect than inactivation of TAZ, although differences between YAP and TAZ may be explained by differences in protein stability and expression. We also identified differences in the transcriptional profiles induced by YAP and TAZ, suggesting that, although they are largely similar, there may be important distinctions between YAP and TAZ as well.

Chapter 1: Introduction to the Hippo pathway and disease implications

The Hippo signaling pathway is important for controlling organ size and tissue homeostasis. Originally identified in *Drosophila melanogaster*, the core components of the Hippo pathway are highly conserved in mammals. In mammals, the canonical Hippo pathway consists of a kinase cascade of Mammalian STE20-like 1/2 (MST1/2) and Large tumor suppressor 1/2 (LATS1/2) (Figure 1.1). When activated, MST forms a heterodimer with the adaptor protein Salvador 1 (SAV1), which enhances MST kinase activity and facilitates MST-LATS interaction (Callus et al., 2006; Tapon et al., 2002). MST then directly phosphorylates Mob1 homolog (MOB1) and LATS. Once phosphorylated, MOB1 binds to the auto-inhibitory region of LATS, enabling LATS phosphorylation and activation (Chan et al., 2005; Praskova et al., 2008). Once activated, LATS phosphorylates the main effectors of the Hippo pathway, transcriptional co-activators Yes-associated protein (YAP) and WW domain containing transcription factor (WWTR1 or TAZ) (Huang et al., 2005; Lei et al., 2008; Oh and Irvine, 2008). LATS kinase activity inhibits YAP/TAZ transcriptional activity by altering YAP/TAZ localization and protein stability.

Phosphorylated YAP/TAZ are sequestered in the cytoplasm by binding to 14-3-3, where they are ubiquitinated and degraded (Liu et al., 2010a; Zhao et al., 2010b; Zhao et al., 2007). Additionally, in *Drosophila*, phosphorylated Yorkie (the *Drosophila* ortholog of YAP/TAZ) is actively excluded from the nucleus in an Exportin 1 (XPO1)-dependent manner (Ren et al., 2010). Conversely, when LATS are inactive, dephosphorylated YAP/TAZ translocate to the nucleus to initiate transcription (Dong et al., 2007; Kanai et al., 2000; Oh and Irvine, 2008; Ren et al., 2010). YAP/TAZ do not contain their own

DNA-binding motifs and initiate transcription by interacting with the DNA-binding transcription factors TEA domain family members 1-4 (TEAD1-4) (Vassilev et al., 2001; Wu et al., 2008; Zhang et al., 2008; Zhao et al., 2008). Through these interactions, YAP/TAZ induce expression of genes regulating proliferation, differentiation, and apoptosis. YAP/TAZ also interact with other transcription factors, including SMAD family members (Smad), p63/p73, Paired box 3 (Pax3), and T-box transcription factor 5 (TBX5) (Ferrigno et al., 2002; Murakami et al., 2005). However, the roles of these transcription factors in mediating the growth promoting activities of YAP/TAZ have not yet been established.

1.1 Regulation of the Hippo pathway

The Hippo pathway can be regulated at many levels. For instance, YAP/TAZ nuclear localization can be modulated by cell contact inhibition (Ota and Sasaki, 2008; Zhao et al., 2007). Neurofibromin 2 (NF2 / Merlin), a tumor suppressor localized near adherens and tight junctions, mediates contact inhibition by recruiting LATS to the cell membrane where LATS is phosphorylated and activated by MST and SAV1 (Yin et al., 2013). In addition, nuclear NF2 inhibits E3 ubiquitin ligase $CRL4^{DCAF1}$-mediated LATS degradation, resulting in LATS accumulation and YAP phosphorylation and inactivation (Li et al., 2014). Several components of adherens and tight junctions, including Angiomotin (AMOT), alpha-catenin (CTNNA1), and Protein tyrosine phosphatase non-receptor 14 (PTPN14), also directly interact with the Hippo pathway. AMOT induces LATS2-mediated phosphorylation of YAP and sequesters YAP/TAZ to tight junctions,

thereby preventing YAP/TAZ from translocating to the nucleus and from initiating any transcriptional activity (Chan et al., 2011; Paramasivam et al., 2011; Wang et al., 2011; Zhao et al., 2010a). However, a recent report found that AMOT can stimulate YAP activity by two mechanisms: by (i) binding YAP in the cytoplasm and preventing its phosphorylation by LATS; and (ii) by forming a transcriptional complex with YAP and TEAD in the nucleus to induce transcription of YAP downstream target genes (Yi et al., 2013). These seemingly contradictory results could be due to tissue or context-specific roles of AMOT in regulating the Hippo pathway. CTNNA1 forms a trimeric complex with phosphorylated YAP and 14-3-3, sequestering YAP to adherens junctions and preventing its dephosphorylation (Schlegelmilch et al., 2011). PTPN14 directly binds and sequesters YAP in the cytoplasm (Liu et al., 2013; Wang et al., 2012b). Together, these findings illustrate how the cell's surroundings tightly regulate the Hippo pathway.

Wnt signaling and extracellular hormones can also regulate YAP/TAZ activity (Azzolin et al., 2012). A large number of hormones act through G protein coupled receptors (GPCRs) to either activate or inhibit YAP/TAZ. Serum, Lysophosphatidic acid (LPA), Sphingosine-1-phosphate (S1P), and thrombin signal via G12/13 and Gq/11 to activate downstream Rho GTPases, modulate the actin cytoskeleton, and activate YAP/TAZ (Yu et al., 2012). Conversely, epinephrine and glucagon signal via Gαs to activate protein kinase A (PKA), modulate the actin cytoskeleton, and induce YAP/TAZ phosphorylation (Yu et al., 2012). Although it is clear that changes in actin cytoskeleton dynamics are important for mediating upstream signals to regulate YAP/TAZ, the full mechanism is unknown. GPCRs play an important role in modulating a wide range of

cellular processes, including cell proliferation and survival, and it is likely that some of these functions are mediated through the Hippo pathway.

1.2 The Hippo pathway in regeneration and development

The Hippo pathway in liver regeneration

The liver has a remarkable ability to regenerate following injury. Although the primary source of new tissue during regeneration is proliferating hepatocytes, hepatocytes are quiescent under normal conditions. MST1/2 inactivation is required to wake hepatocytes out of quiescence, suggesting the Hippo pathway and active YAP/TAZ play a necessary role in initiating regeneration (Avruch et al., 2011). Moreover, hepatocyte-specific *MST1/2* knockout is sufficient to dramatically increase hepatocyte proliferation, resulting in massive liver overgrowth due to aberrant YAP activity (Zhou et al., 2009). In addition, liver overgrowth caused by inducible, liver-specific YAP overexpression is due to an increase in hepatocyte cell number, not cell size, suggesting that the role of MST1/2 inactivation is indeed mediated by YAP/TAZ, and that activation of YAP/TAZ itself is sufficient to initiate cell proliferation in quiescent hepatocytes (Dong et al., 2007). Recent work has also shown that inducing expression of a constitutively active YAP in hepatocytes *in vivo* can cause hepatocytes to dedifferentiate back into progenitor cells, which may have important implications in our understanding of the mechanisms behind liver regeneration (Yimlamai et al., 2014).

YAP protein levels are markedly increased during liver regeneration in humans (Bai et al., 2012; Wang et al., 2012a), as well as during regeneration following

hepatectomy (Apte et al., 2009), bile acid-induced injury (Anakk et al., 2013), and bile duct ligation-induced injury (Bai et al., 2012) in mice. Following bile duct ligation, liver-specific *YAP* knockout mice are more susceptible to injury and show reduced hepatocyte proliferation and increased necrosis, indicating that YAP is required for regeneration (Bai et al., 2012). Deleting *MST1/2* to activate YAP also protects the liver from acetaminophen-induced liver injury (Wu et al., 2013). A recent study found that following a partial hepatectomy in rats, YAP activation is accompanied by MST1/2, LATS1/2, and MOB1 inactivation (Grijalva et al., 2014). However, once the liver reaches its pre-hepatectomy size, MST1/2 activity is restored, which in turn is followed by YAP inactivation (Grijalva et al., 2014). This study highlights the importance of canonical Hippo pathway components in dynamically regulating YAP during regeneration and maintenance of final liver size. In other genetic models, AMOT can increase YAP activity by preventing YAP phosphorylation and increasing YAP-TEAD transcriptional activity, so it is not surprising that liver-specific *AMOT* knockout mice also have reduced cell proliferation and regeneration following toxin-induced injury (Yi et al., 2013). Liver-specific knockdown of CTNNA1 also results in increased YAP activity and liver overgrowth following partial hepatectomy, indicating that CTNNA1 may play a role in inactivating YAP following regeneration (Herr et al., 2014). Taken together, these findings suggest that YAP-TEAD transcriptional activity is activated in response to multiple types of injury and is required for initiating cell proliferation and for complete hepatic recovery. Following complete regeneration, YAP activity is regulated at multiple levels, including by canonical Hippo pathway kinases MST1/2, LATS1/2, and MOB1, as well as CTNNA1, to inactivate YAP and prevent liver overgrowth.

The Hippo pathway in pancreatic development

Intact Hippo signaling is also required for normal pancreatic development. Knock down of YAP is sufficient to block pancreatic progenitor cell proliferation (Zhang et al., 2013). miR-375 can also regulate pancreatic progenitor cell proliferation by inhibiting translation of YAP mRNA via binding to the 3' UTR, further supporting a role for YAP/TAZ in pancreatic development (Zhang et al., 2013). Furthermore, YAP's role in pancreatic development appears to be compartment specific. Pancreas-specific *MST1/2* knockout and ectopic YAP overexpression both result in decreased pancreas size (Gao et al., 2013b; George et al., 2012). Dysregulation of the Hippo pathway does not appear to play a significant role for the endocrine compartment, and *MST1/2* knockout mice have normal fed blood glucose levels (George et al., 2012). However, the exocrine compartment of *MST1/2* knockout mice shows a dramatic increase in cell proliferation, accompanied by a similar increase in cell death (Gao et al., 2013b). The decrease in pancreas size then is primarily due to loss of tissue architecture in the exocrine compartment due to dedifferentiation of acinar cells back into ductal cells (Gao et al., 2013b; George et al., 2012). This appears to be YAP-dependent because deleting a single allele of *YAP* in the *MST1/2* knockout mice results in improved pancreatic growth and structure (Gao et al., 2013b). The difference between the endocrine and exocrine compartments is probably due to expression levels, since YAP is not expressed in the endocrine compartment following differentiation. Nevertheless, it will be interesting to see how YAP becomes differentially regulated in the endocrine compartment, as well as whether TAZ is similarly regulated.

The Hippo pathway in ocular development

YAP/TAZ transcriptional activity is regulated by both mechanical and biochemical signals and plays a critical role in ocular development, regeneration, and disease. For instance, Sveinsson's Chorioretinal Atrophy (SCRA), a rare genetic disease resulting in degeneration of the choroid and retina, is caused by a mutation in *TEAD1* (Kitagawa, 2007). This mutant TEAD1 is defective in YAP/TAZ binding and has no transcriptional activity (Kitagawa, 2007). Interestingly, this work raised the possibility that YAP-TEAD transcriptional activity is important for cell-cell and cell-matrix adhesions, as SCRA is caused by tearing of the retinal pigment epithelium (RPE) (Kitagawa, 2007). Conversely, in other cellular contexts, increased YAP expression is correlated with epithelial to mesenchymal transition (EMT) and the loss of cell-cell junctions. In fact, it has been shown in RPE cells that TAZ-TEAD1 transcriptional activity results in ZEB1 expression, loss of cell-cell contact, and EMT (Liu et al., 2010b). The observation that defective YAP-TEAD transcriptional activity is also correlated with defective cell-cell and cell-matrix adhesions may lead to a more dynamic understanding of the role of YAP in regulating cell-cell interactions. In contrast, *NF2* knockout mice develop cataracts caused by disorganization and accumulation of cells in the lens epithelium due to abnormal tissue growth (Zhang et al., 2010). This is rescued by deleting *YAP*, indicating that this phenotype is dependent on the Hippo pathway. This phenotype aligns with the current understanding of elevated YAP activity resulting in overgrowth, as seen in other tissues and organs.

The Hippo pathway in intestinal regeneration

The Hippo pathway is important for maintaining intestinal homeostasis. The intestinal lining is constantly exposed to a harsh environment and must continually regenerate to replace dying cells. Intestine-specific *YAP* knockout mice show no major effects during development or on normal homeostasis (Cai et al., 2010). However, YAP plays an important role in regeneration following injury. In a Dextran Sodium Sulfate (DSS)-induced colonic regeneration model, YAP protein levels are increased during regeneration. Deleting *YAP* blocks regeneration in this model and results in substantial intestinal damage and increased mortality (Cai et al., 2010), indicating YAP is required for regeneration. However, another study looked at intestinal regeneration following whole body irradiation and found that intestine-specific *YAP* overexpression resulted in impaired regeneration, and intestine-specific *YAP* knockout mice developed hyperplasia (Barry et al., 2013). They also found that expressing constitutively active YAP can suppress growth of colorectal cancer (CRC) xenografts, suggesting YAP acts as a tumor suppressor. These conflicting reports may be due to multiple factors. The role of YAP in regeneration may be injury specific, since the two studies utilized different injury models to induce regeneration. Some differences might also be explained by the involvement of other signaling pathways. For instance, YAP overexpression-induced dysplasia can be blocked by Gamma-Secretase inhibitors, which inhibit Notch signaling (Camargo et al., 2007). In addition, Barry *et al.* found that YAP is silenced in a subset of human CRC, and YAP blocks regeneration by inhibiting Wnt signaling and preventing Dishevelled (DVL) nuclear translocation (Barry et al., 2013; Varelas et al., 2008). Loss of *YAP* results in Wnt hypersensitivity during regeneration, which might cause

hyperplasia (Barry et al., 2013; Tian et al., 2007). Further work is needed to fully understand how these different pathways interface in specific cellular niches and how they may regulate each other during regeneration.

Hippo pathway in cardiomyocyte regeneration

During development, the heart grows dramatically due to cardiomyocyte proliferation. However, within a week after birth, cardiomyocytes stop proliferating and any subsequent growth is due to cardiomyocyte hypertrophy (Ahuja et al., 2007). As such, the adult heart has a limited ability to regenerate following injury. Instead, the myocardium replaces lost cardiomyocytes with fibrotic scar tissue, which reduces heart contractility and function. Interestingly, recent studies have identified a potential role for the Hippo pathway in enhancing cardiomyocyte proliferation following injury. For instance, conditional MST1 overexpression in the heart results in increased cardiomyocyte apoptosis *in vitro* and dilated cardiomyopathy *in vivo* (Yamamoto et al., 2003). Conversely, overexpressing dominant-negative MST1 or LATS2 showed improved cardiac function following either myocardial infarction or ischemia and reperfusion (Odashima et al., 2007; Shao et al., 2014). In addition, SAV1-deficient cardiomyocytes can re-enter the cell cycle and undergo cell division, and *SAV1* heart-specific knockout mice show improved recovery following ischemia with ejection fraction and fractional shortening values comparable to control, non-ischemic mice (Heallen et al., 2013). These reports suggest that Hippo-deficient hearts exhibit increased regenerative potential. *YAP* transgenic mice also show increased regeneration and decreased fibrosis following heart injury (Xin et al., 2013). More specifically,

Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit beta (PIK3CB), a catalytic subunit of PI3K, is a direct target of YAP in promoting cardiomyocyte proliferation (Lin et al., 2014b). Thus, manipulating the Hippo pathway following injury could be key to improving heart regeneration, decreasing fibrosis, and increasing survival.

1.3 Dysregulation of the Hippo pathway in human disease

While YAP/TAZ activity is important for cell proliferation and regeneration, the Hippo pathway must be tightly regulated. Not surprisingly, dysregulation of the Hippo pathway can lead to uncontrolled proliferation, resulting in a wide range of diseases and cancers. The Hippo pathway can become dysregulated by a variety of mechanisms, including *YAP* gene amplification, deletion of upstream Hippo pathway components, mutations in upstream GPCRs, or by crosstalk with other signaling pathways including Wnt signaling (Figure 1.2).

The Hippo pathway in liver cancer

YAP is frequently amplified in hepatocellular carcinoma (HCC) and is required to sustain increased cell proliferation and tumor growth (Zender et al., 2006). Risk factors for HCC include hepatitis infection and exposure to xenobiotics, and these have also been implicated in YAP activation. The Hepatitis B virus X protein (HBx) directly increases YAP expression by enhancing *YAP* gene transcription (Zhang et al., 2012). 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) is a xenobiotic mimic that

activates Constitutive androstane receptor (CAR) to increase YAP protein levels and induce HCC (Kowalik et al., 2011). In addition, GA-binding protein (GABP), involved in antioxidant defense, can directly promote *YAP* transcription (Wu et al., 2013). Increased GABP nuclear localization and YAP expression are both correlated in liver cancer, so it is possible that high GABP levels promote increased YAP expression in HCC (Wu et al., 2013).

Inducing YAP overexpression in a liver-specific transgenic model causes abnormal hepatocyte proliferation and suppresses apoptosis, resulting in increased liver size and HCC (Camargo et al., 2007; Dong et al., 2007). These findings are consistent with knockouts of other Hippo pathway components. One study deleted *MOB1A*, with a heterozygous mutation in *MOB1B*, and found that these mice have an increased lifetime chance of developing HCC (Nishio et al., 2012). Increased liver growth and HCC have also been reported in liver-specific *SAV1* knockout, *NF2* knockout, and *MST1/2* knockout mice (Lee et al., 2010; Lu et al., 2010; Zhang et al., 2010). These findings implicate an important role for the Hippo pathway in controlling liver size and preventing tumorigenesis. In addition, *NF2* knockout mice show reduced tumorigenesis when crossed with liver-specific *AMOT* knockout mice, suggesting the AMOT-YAP interaction is also important for YAP-driven tumorigenesis (Yi et al., 2013). However, much of the current understanding of the Hippo pathway in HCC has been derived from genetic models, and few mutations or deletions in Hippo pathway components have been observed in human HCC (Gao et al., 2013a). While the mouse work has established a clear role for the Hippo pathway in HCC, future work should focus on how the Hippo pathway becomes dysregulated in human HCC.

YAP/TAZ gene fusion in Epithelioid hemangioendothelioma

Epithelioid hemangioendothelioma (EHE) is a rare vascular tumor most commonly found in the lung, bone, and skin. Recently, it has been shown that *YAP/TAZ* chromosome translocations occur in virtually all EHE cases (Flucke et al., 2014). These chromosome translocations result in a fusion protein between either *TAZ* and *Calmodulin binding transcription activator 1* (*CAMTA1*), *TAZ* and *FBJ murine osteosarcoma viral oncogene homolog B* (*FOSB*), or *YAP* and *Transcription factor binding to IGHM enhancer 3* (*TFE3*) (Antonescu et al., 2014; Antonescu et al., 2013; Tanas et al., 2011). While the fusion proteins retain their YAP/TAZ TEAD-binding domains, they are missing key phosphorylation sites required by LATS to inactivate YAP/TAZ, so these fusion proteins may act as constitutively active transcription factors. Although research on the role of YAP/TAZ in EHE is at its infancy, the observation that YAP/TAZ chromosome translocations occur in virtually all cases of EHE strongly suggest that dysregulated YAP/TAZ fusion proteins may act as cancer drivers.

The Hippo pathway in breast cancer

YAP/TAZ activity has been correlated with increased risk of metastasis and reduced survival across all human breast cancer subtypes (Cordenonsi et al., 2011). However, the role of the Hippo pathway in breast cancer progression remains controversial. On one hand, TAZ is highly expressed in invasive breast cancer cell lines and primary breast cancers, and TAZ overexpression is sufficient to induce cell proliferation, transformation, and EMT in breast cancer cell lines (Chan et al., 2008; Lei

et al., 2008). Similarly, overexpressing YAP in breast cancer cell lines induces tumor formation and growth in xenograft experiments (Wang et al., 2012c), and deleting *YAP* prevents tumor growth in an oncogene-induced breast cancer model (Chen et al., 2014b). In addition, Leukemia inhibitory factor receptor (LIFR) has been identified as a tumor suppressor that acts through the Hippo pathway to inactivate YAP both *in vitro* and *in vivo* (Chen et al., 2012). Together, these reports support an oncogenic role for YAP/TAZ. On the other hand, there are conflicting reports that suggest YAP acts as a tumor suppressor. YAP protein expression is decreased in luminal breast cancer tissues, and YAP knockdown in breast cancer cell lines actually enhances tumor migration, invasion, and tumor growth in nude mice (Yuan et al., 2008). A recent study reported that hyperactivation of YAP alone is not sufficient to drive mammary tumorigenesis *in vivo*, and YAP-induced oncogenic growth may be dependent on the presence of additional driving mutations or amplifications (Chen et al., 2014b). Additional work is needed to determine whether these conflicting reports may be due to cell type-specific differences.

The Hippo pathway in lung cancer

YAP/TAZ are both highly expressed in non-small cell lung cancer (NSCLC) in humans, and knockdown of either YAP or TAZ in NSCLC cells is sufficient to suppress proliferation, invasion, and tumor growth in mice (Wang et al., 2010; Zhou et al., 2011b). High YAP expression is correlated with advanced stage, lymph node metastasis, and decreased survival (Wang et al., 2010). In fact, it has been shown that knockdown of either YAP or TAZ is sufficient to decrease cell migration *in vitro* and metastasis *in vivo*,

and expression of constitutively active YAP is sufficient to drive lung cancer progression *in vivo* (Lau et al., 2014). However, although these studies strongly point towards an oncogenic function for YAP/TAZ, the mechanisms by which YAP/TAZ become dysregulated in NSCLC progression was not known until recently.

Overexpressing MST1 is sufficient to inhibit cell proliferation and apoptosis in NSCLC cells (Xu et al., 2013). This is most likely due to MST activation of LATS, thereby preventing YAP/TAZ nuclear localization. LATS1 protein levels are frequently decreased in NSCLC tissues, and loss of LATS1 expression is correlated with advanced stage, lymph node metastasis, and decreased survival (Lin et al., 2014a). In addition, other non-canonical Hippo pathway components have also been identified to interact with YAP/TAZ in lung cancer. Vestigial-like family member 4 (VGLL4) is frequently down-regulated in lung cancer, and expressing VGLL4 in lung cancer cells suppresses cell proliferation and tumor growth in mice by competitively inhibiting YAP-TEAD binding and transcriptional activity (Zhang et al., 2014a). Another study found that high YAP expression was correlated with increased AXL receptor tyrosine kinase (AXL) expression in lung adenocarcinomas, and that knocking down YAP also resulted in loss of AXL, proliferating cell nuclear antigen (PCNA), and matrix metalloproteinase-9 (MMP-9) (Cui et al., 2012). This study further confirmed that knocking down YAP inhibits proliferation and invasion of lung cancer cells, an effect which is potentially mediated through AXL. Finally, miR-135b expression increases lung cancer metastasis by targeting LATS2, and inhibiting miR-135b suppresses tumor growth and metastasis (Lin et al., 2013). Expression of miR-135b is regulated by DNA demethylation and nuclear factor-kappa B (NFKb) signaling, raising the possibility that inflammatory and epigenetic

modifications may regulate expression of miR-135b, thereby resulting in LATS2 inhibition, YAP/TAZ nuclear translocation, and cancer progression (Lin et al., 2013).

The Hippo pathway in Malignant mesothelioma

Malignant mesothelioma is a rare cancer of the mesothelium, the lining which covers many of the body's internal organs, and often comes with a poor prognosis. Recent work has found that homozygous deletion or inactivating mutations in *NF2*, *SAV1*, or *LATS2* are frequently observed in human malignant mesothelioma tissues and cell lines (Mizuno et al., 2012; Murakami et al., 2011). Moreover, Ajuba LIM protein (AJUBA) can inactivate YAP through signaling via LATS, and down-regulation of AJUBA has also been associated with malignant mesothelioma (Tanaka et al., 2013). These deletions or mutations contribute to increased YAP protein levels and aberrant YAP-TEAD transcriptional activity, which drive increased cell proliferation and anchorage-independent growth by up-regulating the cell cycle-promoting Cyclin D1 and Forkhead box M1 (Mizuno et al., 2012). Knocking down YAP in malignant mesothelioma cells is sufficient to inhibit cell proliferation and anchorage-independent growth (Mizuno et al., 2012). Together, these findings strongly implicate dysregulation of the Hippo pathway in malignant mesothelioma and identify YAP as a potential therapeutic target. As cases of malignant mesothelioma are primarily associated with asbestos exposure, it may be informative to determine whether there is something about asbestos that is pre-inclined to inducing mutations in Hippo pathway components.

The Hippo pathway in pancreatic cancer

Pancreatic ductal adenocarcinoma (PDAC) has one of the worst prognoses of all cancers because the patient often does not experience any symptoms until the cancer has reached an advanced stage. PDAC tissues often have increased YAP expression and nuclear localization, and elevated YAP expression is correlated with poor prognosis (Diep et al., 2012). Moreover, in pancreatic cancer cells, YAP knockdown results in reduced proliferation and reduced anchorage-independent growth, suggesting YAP may play an important role in PDAC progression (Diep et al., 2012). These findings are supported by work done in *KRAS* transgenic mice. *KRAS* is frequently mutated in PDAC, but in a mouse model expressing mutated *KRAS*, deleting *YAP* is sufficient to prevent PDAC. In addition, deleting *YAP* in pancreatic cancer cells harboring the mutant *KRAS* is sufficient to prevent proliferation and growth in mice (Zhang et al., 2014b). A similar study found that in an inducible KRAS-driven PDAC mouse model where removal of KRAS resulted in complete tumor regression, some mice later developed spontaneous tumors due to YAP amplification and increased YAP-TEAD2 transcriptional activity (Kapoor et al., 2014). This finding suggests that KRAS-driven tumors may acquire additional mechanisms to further increase proliferation and growth, and YAP may play an important role in enabling PDAC to escape KRAS addiction.

The Hippo pathway in Kaposi sarcoma

YAP/TAZ play a driving role in Kaposi sarcoma (KS), a tumor caused by the Kaposi sarcoma-associated herpesvirus (KSHV). KS results in cutaneous lesions which can spread throughout the skin, mouth, gastrointestinal, and respiratory tracts. Tissue

samples from human KS patients show elevated levels of YAP/TAZ (Liu et al., 2014). Recently, it was shown that KSHV encodes a viral GPCR (vGPCR), which signals through Gq/11 and G12/13 to RhoA, inactivating LATS1/2 and activating YAP/TAZ (Liu et al., 2014). In addition, cells overexpressing vGPCR failed to grow in a xenograft mouse model when YAP/TAZ were depleted, indicating that YAP/TAZ are necessary for KSHV-induced tumorigenesis.

The Hippo pathway in Uveal melanoma

Uveal melanoma (UM) is the most common type of eye cancer in adults, with approximately 80% of UM cases characterized by activating mutations in either *GNAQ* or *GNA11*. Although overexpression of mutant Gq/11 is sufficient to transform melanocytes (Van Raamsdonk et al., 2010), the signaling events downstream of Gq/11 were unknown. Two studies showed that Gq/11 can activate YAP by inhibiting LATS1/2 and disrupting AMOT-YAP interaction (Feng et al., 2014; Yu et al., 2014). Importantly, both papers demonstrate that treating UM with Verteporfin, a drug which blocks YAP-TEAD interaction (therefore inhibiting YAP transcriptional activity), is sufficient to inhibit UM tumor growth in mice (Liu-Chittenden et al., 2012; Van Raamsdonk et al., 2010). This is an important finding not only for treating UM, but also has broad implications for how YAP may be involved in other GPCR-associated cancers.

The Hippo pathway in Renal cell carcinoma

YAP has also been implicated in renal cell carcinoma (RCC). A recent report found that the *LATS1* promoter is frequently methylated in RCC, resulting in down-

regulation of LATS1 and increased YAP activity (Chen et al., 2014a). Indeed, RCC tissues show elevated levels of YAP, and knocking down YAP in RCC cell lines causes decreased cell proliferation and increased apoptosis (Cao et al., 2014). Although more work is needed to assess the role of YAP in RCC initiation and progression *in vivo* and whether YAP is essential for RCC survival, this report raises the exciting possibility that YAP may be a useful therapeutic target for RCC.

The Hippo pathway in Polycystic kidney disease

Polycystic kidney disease (PKD) is a life-threatening disease caused by cyst formation throughout the kidneys, and is frequently caused by inactivating mutations in either *PKD1* or *PKD2* (Tian et al., 2007). Interestingly, YAP and TAZ appear to serve different functions in PKD progression. The planar cell polarity component Four-jointed (Fjx1) is required for regeneration following tubular epithelial injury, but is decreased in a *PKD1*-inducible knockout model for PKD (Happe et al., 2011). Fjx1 is a transcriptional target of YAP, and Fjx1 and YAP expression are both increased during regeneration following injury in both control and *PKD1* knockout mice. However, in *PKD1* knockout mice, YAP nuclear localization and transcriptional activity continued to persist after recovery, resulting in cyst formation (Happe et al., 2011). Increased YAP expression was also observed in human PKD patients (Happe et al., 2011). Thus, while YAP seems to play a role in kidney recovery following injury, sustained signaling may cause PKD. TAZ appears to have a more direct contribution to PKD. TAZ forms a complex with Polycystin-2 (PC2, the protein product of *PKD2*), thereby targeting it for ubiquitination and degradation. *TAZ* knockout results in PC2 accumulation, leading to

PKD (Tian et al., 2007), and also results in the down-regulation of other genes necessary for proper cilia development and function (Hossain et al., 2007). The lack of functional cilia also contributes to cyst formation. In fact, *TAZ* knockout mice begin developing cysts as early as embryonic day 15.5, possibly due to a combination of these factors (Makita et al., 2008). This phenotype was also seen in a mouse model with *TAZ* conditionally knocked out in nephrons (Reginensi et al., 2013).

The Hippo pathway in colorectal cancer

Although the most common mutations in CRC involve *Adenomatous polyposis coli* (*APC*) and dysregulated beta-catenin signaling, YAP/TAZ may be required downstream mediators of these mutations. YAP/TAZ are reported to be degraded by the beta-catenin destruction complex, along with APC, Axin, and Glycogen synthase kinase 3 (GSK3) (Azzolin et al., 2014). Beta-catenin is required to recruit TAZ to the beta-catenin destruction complex, and the absence of Wnt signaling results in both beta-catenin and YAP/TAZ cytoplasmic sequestration, phosphorylation, and degradation (Azzolin et al., 2012). Cytoplasmic YAP/TAZ can also directly interact with DVL and beta-catenin, inhibiting DVL phosphorylation and preventing beta-catenin nuclear translocation (Barry et al., 2013). Conversely, activation of Wnt signaling results in both beta-catenin and TAZ accumulation, and YAP/TAZ co-transcriptional activity is required for many of the Wnt transcriptional responses (Azzolin et al., 2012). In fact, one study found that beta-catenin-driven tumors require YAP and TBX5 to induce expression of genes required to inhibit apoptosis and promote tumor survival (Rosenbluh et al., 2012). Finally, beta-catenin can interact with TCF/LEF to directly

induce YAP gene transcription (Konsavage et al., 2012). It is clear that crosstalk between the Hippo and Wnt signaling pathways plays an important role in CRC, and this must be taken into consideration when therapeutically targeting either pathway.

YAP is often overexpressed in CRC, and YAP/TAZ activity is correlated with decreased survival (Yu et al., 2015). *LATS1* promoter methylation has also been reported in CRC, which may lead to increased YAP activity (Wierzbicki et al., 2013). In mice, inducing YAP overexpression in the intestine results in dysplasia after two days, although the intestine regenerates once induction is stopped (Camargo et al., 2007). Similar phenotypes were also seen in *MST1/2* and *SAV1* knockout mice, which developed adenomas after 13 weeks and polyps after 13 months, respectively (Cai et al., 2010; Zhou et al., 2011a). Both of these phenotypes were blocked by deleting *YAP*, indicating that these pathologies are YAP-dependent. In addition, one report suggests that YAP may play an important role in causing CRC cells to become dormant during chemotherapy treatment and active during relapse (Touil et al., 2014). Cells resistant to 5-fluorouracil (5FU) express high levels of YAP, which becomes phosphorylated and cytoplasmic when the cells are exposed to 5FU, causing the cells to enter quiescence. Increased YAP protein levels were also seen in human CRC liver metastases and were correlated with CRC relapse (Touil et al., 2014). Although the authors did not show whether removing 5FU causes increased YAP nuclear localization and cell proliferation, these findings are highly significant and may hugely impact the paradigm for treating CRC and preventing relapse.

The Hippo pathway in multiple myeloma

The Hippo pathway plays an important role in regulating lymphocyte apoptosis. YAP acts as a tumor suppressor in several hematological cancers, including multiple myeloma (MM), lymphoma, and leukemia (Cottini et al., 2014). These cancers are typically characterized by genetic instability and inactivating mutations in *Tumor protein p53* (*TP53*). In human MM patient samples, *YAP* is also frequently deleted or down-regulated (Cottini et al., 2014). YAP interacts with ABL1 to induce p53-independent apoptosis, and inhibiting MST1 in MM cells is sufficient to up-regulate YAP protein levels and induce apoptosis, both *in vitro* and *in vivo* (Cottini et al., 2014). This report raises the possibility that YAP may act as a tumor suppressor and proposes a novel therapeutic strategy for targeting the Hippo pathway in hematological cancer. Little is known about YAP/TAZ in hematological cells, and any role of YAP/TAZ as tumor suppressors would challenge the current paradigm that YAP/TAZ act as oncogenes.

The Hippo pathway in the nervous system

The Hippo pathway is involved in several nervous system tumors. Loss of function mutations in *NF2* causes Neurofibromatosis Type 2 (NF2), a genetic disorder characterized by the development of schwannomas and meningiomas with increased YAP expression and nuclear localization (Schulz et al., 2014; Striedinger et al., 2008). NF2 inhibits YAP activity by promoting LATS activation and inhibiting LATS ubiquitination and degradation (Li et al., 2014; Yin et al., 2013). Loss of function mutations in *NF2* results in increased LATS degradation and YAP accumulation, so loss of NF2 and subsequent tumor growth could be due to aberrant YAP activity. In the

central nervous system, NF2 expression is also significantly reduced in human malignant gliomas, and expression of NF2 has been shown to inhibit human glioma growth both *in vitro* and *in vivo* (Lau et al., 2008). Likewise, YAP is highly expressed in many human brain tumors including infiltrating gliomas, and YAP overexpression promotes glioblastoma growth *in vitro* (Orr et al., 2011).

In non-cancer neurological diseases, Hippo pathway components are also highly involved. For instance, it is reported that YAP/TAZ mediate gene transcription induced by ABPP, the precursor of Amyloid beta which is thought to drive Alzheimer's disease (Orcholski et al., 2011). In addition, MST1 is a key mediator of Amyotrophic Lateral Sclerosis (ALS). MST1 activity is increased in motor neurons from SOD1(G93A) mice, an ALS mouse model. MST1 activates p38 and Caspase-3 and -9, resulting in autophagosome accumulation and motor neuron death. When MST1 is knocked out in these mice, they show increased motor neuron viability, delayed symptom onset, and extended survival, although it is not clear whether YAP or TAZ are involved in this phenotype (Lee et al., 2013). These findings demonstrate the importance of the Hippo pathway in some neurodegenerative diseases and can hopefully be expanded in the near future.

The Hippo pathway in Arrhythmogenic cardiomyopathy and Holt-Oram syndrome

The Hippo pathway has also been implicated in several heart diseases. Arrhythmogenic right ventricular cardiomyopathy (ARVC) is characterized by thinning of the right ventricular walls, replacement of the myocardium with fibroadipocytes, and arrhythmias. ARVC is caused by the loss of intact desmosomes. Recent work has

shown that MST1/2, LATS1/2, and YAP are phosphorylated in human ARVC hearts, as well as in knockout mouse models for obligatory desmosome components Desmoplakin (DSP) and Junction plakoglobin (JUP) (Chen et al., 2014c). In addition, knock down of LATS1/2 or overexpressing a constitutively active YAP mutant in cardiomyocytes results in adipogenesis, further supporting a causal role for the Hippo pathway in ARVC (Chen et al., 2014c). The Hippo pathway is also involved in Holt-Oram syndrome, which consists of heart defects and abnormalities of the upper limbs. TBX5, which is essential for cardiac and limb development, is often mutated in Holt-Oram patients (Basson et al., 1997). TBX5 normally interacts with YAP/TAZ, but mutations in TBX5 prevent its binding to TAZ and results in a congenital heart defect called Tetralogy of Fallot (TOF) (Murakami et al., 2005). Taken together, the Hippo pathway plays an important role in heart development. Future work is needed to determine whether the Hippo pathway can be manipulated or therapeutically targeted to improve regeneration following injury.

1.4 Areas of future inquiry

YAP/TAZ and mechanosensing

Mechanical forces play a central role in regulating gene expression and cell differentiation, especially during development and organ growth. Although it is clear that the Hippo pathway is regulated by changes in extracellular matrix (ECM) stiffness and actin cytoskeleton dynamics, an important question in the field is how the cell is able to translate these mechanical signals into biochemical signals to regulate YAP/TAZ (Dupont, 2016; Meng et al., 2016). For example, changes in ECM stiffness have already

been associated with tumor progression (Ahmadzadeh et al., 2017) and changes in YAP/TAZ activity. While the tyrosine kinase inhibitor Lapatinib is used to treat HER2-positive breast cancers, increased ECM rigidity is associated with increased YAP/TAZ nuclear localization and decreased Lapatinib sensitivity (Lin et al., 2015). However, this sensitivity can be reversed even at increased matrix stiffness by knockdown of either YAP or TAZ. Together, these observations highlight the mechanisms by which changes in ECM stiffness lead to physiologically and therapeutically significant outcomes, and in which YAP/TAZ play a central role.

There are additional reports of how YAP/TAZ activation by mechanical signals plays an important role in regeneration and disease, albeit in an organ or cell-type specific manner. For instance, in pulmonary hypertension, ECM stiffening of the diseased lung results in YAP/TAZ activation, thereby promoting YAP/TAZ-dependent expression of Glutaminase (GLS1), anabolic biosynthesis, and cell proliferation and migration (Bertero et al., 2016). However, in modelling various kidney diseases, YAP/TAZ were actually inactivated by increasing ECM stiffness in podocytes (Rinschen et al., 2017). When grown on soft ECM, YAP was activated and expression of downstream target genes was increased, similar to what has been observed in glomerular disease patients.

Moreover, it has been shown that manipulating YAP/TAZ activity is sufficient to induce cellular changes overriding these mechanical signals. Over-expressing a constitutively active TAZ mutant in mouse fibroblasts is sufficient to promote spheroid formation and induce expression of the pro-fibrotic factors Connective tissue growth factor (CTGF), Endothelin-1 (Et-1), and Plasminogen activator inhibitor 1 (PAI-1)

(Jorgenson et al., 2017). In addition, when cultured on 2D matrices, a constitutively active TAZ mutant enhances contractile force generation, indicating that TAZ may play a role in active remodeling of the ECM, initiating fibroblast matrix remodeling, and fibrosis. This is further supported by the observation that YAP/TAZ show increased nuclear localization in keloid fibrotic tissue (Aramaki-Hattori et al., 2017).

YAP/TAZ can also regulate other signaling pathways in response to mechanical signals. In response to high ECM stiffness, YAP/TAZ induce expression of Delta-like ligands to inhibit Notch signaling and cell differentiation and promote stemness in epidermal stem/progenitor cells (Totaro et al., 2017). Conversely, pharmacologically disrupting the actin cytoskeleton or low ECM stiffness results in YAP/TAZ sequestration in the cytoplasm, induction of Notch signaling, and differentiation. However, this may be Notch-specific, as TGF signaling can be restricted in epithelial cells independent of the Hippo pathway in response to increasing cell density or cell polarization (Nallet-Staub et al., 2015). In addition, the ECM proteoglycan Agrin also translates ECM stiffness to YAP through integrin-focal adhesion and Lrp4 mediated-signaling pathways (Chakraborty et al., 2017). With increasing ECM stiffness, Agrin activates YAP in a manner opposed to NF2 or LATS1/2. These findings serve as a reminder of how tightly regulated YAP/TAZ activity is. Even though there has been much progress regarding how mechanical signals translate to regulate YAP/TAZ, and how YAP/TAZ in turn affects the surrounding ECM, this remains an exciting area of inquiry.

YAP/TAZ and regulation of TEAD1-4

As previously discussed, YAP and TAZ do not have their own DNA binding

motifs, so they induce gene expression through interactions with the DNA transcription factors TEAD1-4. This essential role for TEAD1-4 in translating YAP/TAZ activity to gene expression is evident in osteosarcoma, where knockdown of TEAD1 is sufficient to suppress YAP/TAZ target gene expression, cell proliferation, invasion, and promote apoptosis (Chai et al., 2017). However, although TEAD1-4 are well established to be critical for YAP/TAZ activity, how they are regulated has not been well studied.

Recent work has reported that TEAD1-4 are S-palmitoylated at a highly-conserved cysteine, and that palmitoyation is important for TEAD1-4 stability and binding to YAP/TAZ; when these cysteines are mutated to alanine, TEAD1-4 protein expression is almost completely abolished (Chan et al., 2016; Noland et al., 2016). Moreover, TEAD1-4 localization can also be regulated by environmental stress. Stresses such as osmotic stress, high cell density, and cell detachment induce TEAD1-4 cytoplasmic localization in a p38-dependent manner (Lin et al., 2017). Further, when TEAD1-4 are sequestered in the cytoplasm, YAP/TAZ are unable to translocate to the nucleus, even when dephosphorylated. These studies highlight the pharmacological potential of targeting TEAD1-4 palmitoylation or localization as a novel approach to disrupt YAP/TAZ activity.

Deubiquitinase regulation of YAP/TAZ

While much of the focus on YAP/TAZ regulation has been on phosphorylation, recent work has shifted to deubiquitinases. The deubiquitinase YOD1 was identified as an important regulator of LATS1 (Kim et al., 2017). Identified via an siRNA screen, YOD1 deubiquitinates ITCH, an E3 ligase of LATS1, which results in LATS1

degradation and increased YAP/TAZ stability. Inducing YOD1 expression in a transgenic mouse model is sufficient to cause YAP/TAZ-dependent hepatomegaly. Additionally, the deubiquitinating enzyme USP9X was identified from proteomic studies as a strong interactor with LATS2 (Toloczko et al., 2017). Knockdown of USP9X results in destabilization of LATS2, induction of YAP/TAZ target gene expression, and epithelial-to-mesenchymal transition (EMT) and increased anchorage-independent growth in MCF10A cells. While our understanding of the signal transduction leading to YAP/TAZ phosphorylation and inactivation is somewhat comprehensive, the next shift will be towards more fully elucidating the mechanisms and kinetics by which the Hippo pathway and YAP/TAZ are dephosphorylated and activated.

1.5 Concluding remarks

The Hippo signaling pathway plays an important role in regulating key cellular functions, including cell proliferation, apoptosis, and differentiation. Recognized for its driving contribution in a wide variety of diseases and cancers (Table 1), research into identifying new ways to therapeutically target the Hippo pathway has expanded tremendously in recent years. A screen of FDA approved drugs found that Verteporfin can bind YAP and prevent YAP-TEAD interaction (Liu-Chittenden et al., 2012). As discussed earlier, Verteporfin can block tumor growth in UM, as well as suppress tumor growth in a *NF2* knockout or YAP overexpression liver cancer model (Feng et al., 2014; Liu-Chittenden et al., 2012; Yu et al., 2014). A cell-based screen identified dobutamine, a beta-adrenergic receptor agonist, as another YAP inhibitor, inducing LATS-

independent YAP phosphorylation (Bao et al., 2011). A recent report designed cyclic YAP-like peptides to prevent YAP-TEAD interaction, although it remains to be seen whether these peptides can block YAP transcriptional activity (Zhou et al., 2014). Finally, the finding that some GPCR ligands induce YAP phosphorylation opens the possibility that YAP activity may be therapeutically altered by modulating GPCR signaling (Yu et al., 2012). This is an exciting time when our knowledge of the Hippo field is expanding tremendously and will hopefully lead to the development of specific drugs to manipulate YAP/TAZ activity.

However, many important and fundamental questions still remain. Further elucidating the crosstalk between the Hippo pathway and Wnt, TGF-Beta, Notch, Ras, mTOR, and Sonic hedgehog signaling remains a priority. Additional areas of future research include further elucidating whether YAP/TAZ always function as oncoproteins, or whether they also have context-specific tumor suppressing functions. Finally, it will be important to dissect whether YAP and TAZ are differentially regulated and whether they activate different transcriptional profiles. Addressing these questions may help open the door to the next wave of discoveries in the Hippo field.

1.6 Acknowledgments

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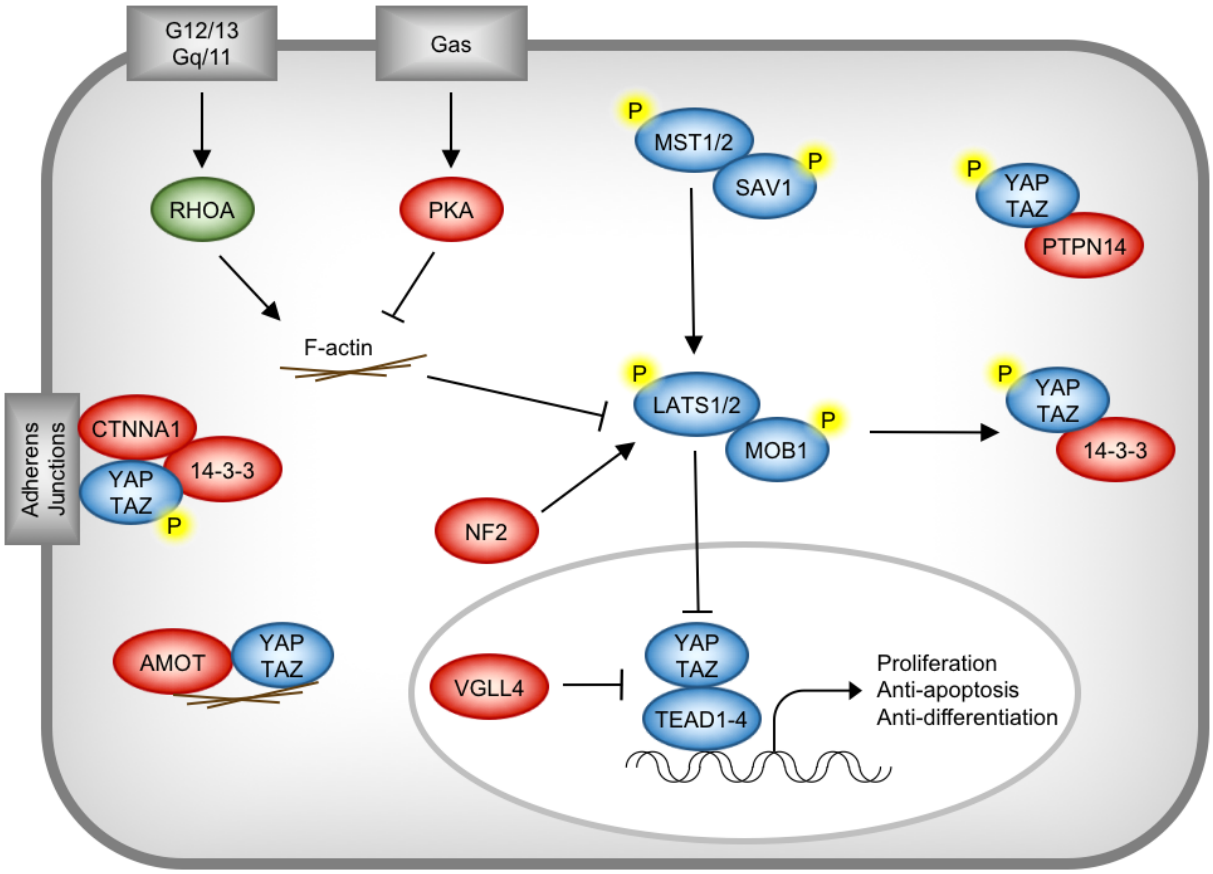

Figure 1.1: The Hippo signaling pathway.
When the Hippo pathway is activated, mammalian STE20-like 1/2 (MST1/2) in complex with Salvador 1 (SAV1) phosphorylate and activate large tumor suppressor 1/2 (LATS1/2) and Mob1 homolog (MOB1). When activated, LATS1/2 phosphorylates Yes-associated protein (YAP)/WW domain-containing transcription factor (WWTR1 or TAZ), the primary effectors of the Hippo pathway. When phosphorylated, YAP/TAZ are sequestered in the cytoplasm or degraded. When YAP/TAZ are dephosphorylated, they translocate to the nucleus where they interact with TEA domain family members 1–4 (TEAD1–4) to induce transcription and promote cell proliferation and inhibit apoptosis. Arrows and blunt ends indicate activation and inhibition, respectively.

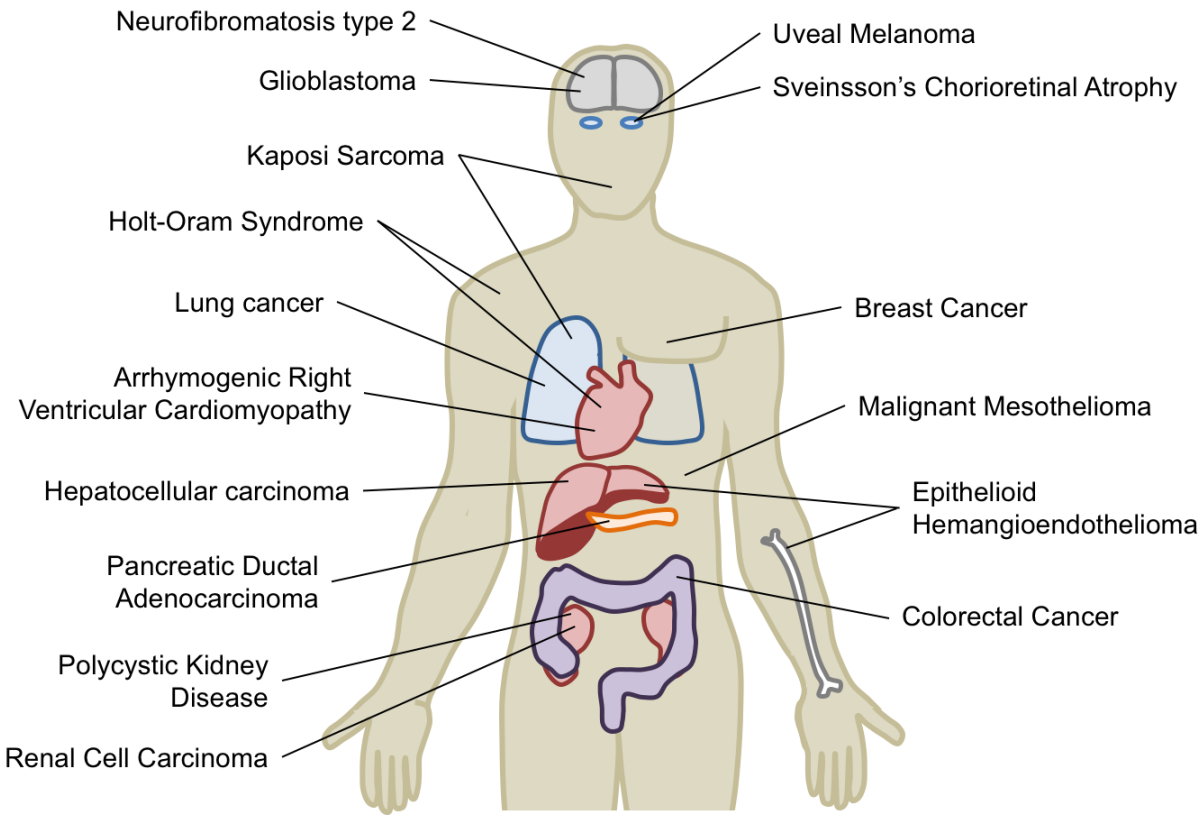

Figure 1.2: Disease implications of the Hippo pathway.
Dysregulation of the Hippo pathway has been implicated in numerous cancers and diseases throughout the body. Dysregulation may be due to genetic inactivation of core pathway components or amplification or gene fusion of the downstream effectors Yes-associated protein (YAP) and WW domain-containing transcription factor (WWTR1 or TAZ). Here we briefly summarize some of the diseases covered in this review.

Table 1.1: Diseases characterized by dysregulated Hippo signaling.

Disease	YAP/TAZ status in humans	Known genetic alterations in humans	Mouse genetic models	References
Diseases characterized by increased YAP expression				
Hepatocellular Carcinoma (HCC)	Increased YAP expression	*YAP* amplification	YAP overexpression, liver-specific *SAV1, NF2, MST1/2* knockout mice develop HCC	(Camargo et al., 2007; Dong et al., 2007; Lee et al., 2010; Lu et al., 2010; Zender et al., 2006; Zhang et al., 2010)
Lung cancer	Increased YAP/TAZ expression is correlated with lymph node metastasis, decreased survival	Loss of LATS1 is correlated with lymph node metastasis, decreased survival	YAP/TAZ knockdown is sufficient to suppress tumor growth; expressing constitutively active YAP is sufficient to drive cancer progression	(Lau et al., 2014; Lin et al., 2014a., Wang et al., 2010)
Pancreatic Ductal Adenocarcinoma (PDAC)	Increased YAP expression		*YAP* knockout prevents PDAC growth	(Diep et al., 2012)
Colorectal Cancer (CRC)	YAP/TAZ are often overexpressed; increased YAP/TAZ is correlated with decreased survival	Frequently caused by mutations in *APC* that may activate YAP/TAZ; *LATS1* promoter is frequently methylated	YAP overexpression, *SAV1*, and *MST1/2* knockout mice develop CRC	(Cai et al., 2010; Camargo et al., 2007; Wierzbicki et al., 2013; Yu et al., 2015; Zhou et al., 2011a)
Renal Cell Carcinoma (RCC)	Increased YAP expression	LATS1 is frequently methylated, resulting in reduced LATS1 expression		(Cao et al., 2014; Chen et al., 2014a)
Neurofibromatosis type 2 (NF2)	Increased YAP expression	Loss of function mutations in *NF2*		(Schulz et al., 2014; Striedinger et al., 2008)

Table 1.1: Diseases characterized by dysregulated Hippo signaling. (continued)

Disease	YAP/TAZ status in humans	Known genetic alterations in humans	Mouse genetic models	References
Diseases characterized by increased YAP expression				
Polycystic Kidney Disease (PKD)	Increased YAP expression	Caused by mutations in *PKD1* or *PKD2*	Sustained YAP activity following injury in *PKD1* knockout mice results in cysts; *TAZ* knockout results in improper cilia development, cyst formation, and PKD	(Happe et al., 2011; Tian et al., 2007)
Diseases characterized by changes in YAP expression				
Breast cancer	Increased YAP/TAZ activity is correlated with increased risk of metastasis, decreased survival; YAP protein levels are decreased in luminal breast cancer tissues		Overexpressing YAP induces tumor formation	(Cordenonsi et al., 2011; Wang et al., 2012c; Yuan et al., 2008)
Diseases characterized by decreased YAP expression				
Multiple Myeloma (MM)		YAP is frequently deleted or down-regulated	Inhibiting MST1 to up-regulate YAP is sufficient to induce apoptosis *in vivo*	(Cottini et al., 2014)
Diseases characterized by chromosome translocations				
Epithelioid Hemangioendothelioma (EHE)		Chromosome translocations between either *TAZ-CAMTA1, TAZ-FOSB*, or *YAP-TFE3*		(Antonescu et al., 2014; Antonescu et al., 2013; Tanas et al., 2011)

Table 1.1: Diseases characterized by dysregulated Hippo signaling. (continued)

Disease	YAP/TAZ status in humans	Known genetic alterations in humans	Mouse genetic models	References
Diseases characterized by mutations or genetic alterations in other Hippo pathway components				
Malignant Mesothelioma		Deletion or inactivating mutations in *NF2, SAV1*, or *LATS2* are common		(Mizuno et al., 2012; Murakami et al., 2011)
Kaposi Sarcoma (KS)	KSHV encodes a vGPCR which activates YAP/TAZ		YAP/TAZ knockdown prevents KSHV-induced tumorigenesis	(Liu et al., 2014)
Uveal Melanoma (UM)		Caused by mutations in *GNAQ* or *GNA11*	Treating UM cells with Verteporfin prevents tumor growth	(Feng et al., 2014; Yu et al., 2014)
Sveinsson's Chorioretinal Atrophy (SCRA)		Caused by mutations in *TEAD1* which prevent YAP-TEAD interaction		(Kitagawa, 2007)
Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC)	YAP is phosphorylated	Caused by mutations in desmosome components *DSP, JUP, DSC2, DSG2,* and *PKP2*	LATS1/2 knockdown or expressing constitutively active YAP causes ARVC; *DSP* or *JUP* KO mice show increased MST1/2, LATS1/2, and YAP phosphorylation	(Chen et al., 2014c)
Holt-Oram Syndrome		Caused by mutations in *TBX5*, which prevents binding to TAZ		(Basson et al., 1997; Murakami et al., 2005)

1.7 References

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Chapter 2: The Hippo pathway regulation of desmosome expression

2.1 Introduction

The Hippo pathway is an important regulator of cell proliferation and apoptosis. Hippo pathway activity can be modulated by changes in G-Protein-Coupled Receptor (GPCR) signaling (Yu et al., 2012), remodeling of the actin cytoskeleton (Aragona et al., 2013), and by interactions with tight-junction associated proteins Neurofibromatosis 2 (NF2) (Zhang et al., 2010), Angiomotin (AMOT) (Zhao et al., 2010), and alpha-catenin (CTNNA1) (Schlegelmilch et al., 2011; Silvis et al., 2011). The Hippo pathway consists of a kinase cascade of Mammalian STE20-like 1/2 (MST1/2) and its binding partner Salvador homolog 1 (SAV1), and Large tumor suppressor 1/2 (LATS1/2) with its co-activator Mob1 homolog (MOB1). Once activated, MST1/2 interacts with SAV1 and MOB1 to phosphorylate and activate LATS1/2. Activated LATS1/2 then phosphorylates the downstream effectors of the Hippo pathway, Yes associated protein (YAP) and WW domain containing transcription factor (WWTR1 or TAZ). Phosphorylated YAP/TAZ are bound by 14-3-3 and sequestered in the cytoplasm, where they are ubiquitinated and degraded. Conversely, when the Hippo pathway is inactive, dephosphorylated YAP/TAZ translocate to the nucleus where they interact with TEA domain transcription factors 1-4 (TEAD1-4) to induce expression of genes promoting cell proliferation and cell growth (Yu and Guan, 2013).

During development and regeneration, mechanical forces and environmental cues play a critical role in determining final organ size. YAP/TAZ play a critical role in mediating the cellular response to at least one of these signals, cell contact inhibition

(Zhao et al., 2007). For example, ACHN cells, which are derived from a metastatic human renal adenocarcinoma with deletion of SAV1, no longer respond to cell contact inhibition; they continue proliferating regardless of confluency, growing on top of each other even at low density. However, when these cells are forced to express a dominant negative YAP mutant, they become sensitive to cell contact inhibition and only grow as a single monolayer (Zhao et al., 2007). Thus, modulating YAP by itself is sufficient to disrupt the cell's response to cell contact inhibition.

Similarly, YAP/TAZ overexpression or hyper-activation has been correlated with uncontrolled proliferation and tumorigenesis (Plouffe et al., 2015). A study looking at core biopsy specimens from human patients with colonic adenocarcinoma, lung adenocarcinoma, and ovarian cystadenocarcinoma found that YAP expression was significantly increased in neoplastic tissues relative to controls (Steinhardt et al., 2008). Subsequent studies have also demonstrated that YAP/TAZ overexpression is sufficient to induce cells to undergo an Epithelial-Mesenchymal Transition (EMT) (Diepenbruck et al., 2014; Lei et al., 2008; Liu et al., 2010). During EMT, cells assume a mesenchymal-like morphology, lose their intercellular junctions, overcome cell contact inhibition, and acquire the ability to migrate. EMT has been associated with giving rise to cells with cancer stem cell-like properties and increased metastatic potential in vivo (Mani et al., 2008; Polyak and Weinberg, 2009; Sanchez-Tillo et al., 2012; Savagner, 2001).

As mentioned, one of the early stages of EMT progression is the loss of intercellular junctions, including the loss of desmosomes. Desmosomes are large intercellular complexes responsible for maintaining cell-cell adhesion and providing the resistance necessary for tissues to withstand mechanical stress by anchoring to

intermediate filaments. They are most prevalent in epithelial and cardiac muscle cells, and their essential role in maintaining tissue integrity is highlighted by several skin and heart diseases, including Pemphigus Vulgaris of the skin (which is characterized by severe blistering of the skin and mucous membranes) and Arrhythmogenic Right Ventricular Dysplasia and Cardiomyopathy (ARVD/C) of the heart (one of the primary causes of arrhythmias), which are caused by mutations in desmosome components. In addition, genetic mouse models with knockouts of core desmosome components are almost all either embryonic lethal or display severe heart defects, blistering, and hair loss (reviewed in (Schmidt and Koch, 2007)).

Although there are structural similarities between desmosomes and other intercellular junctions, there are important regulatory and functional differences as well. Briefly, tight junctions establish an epithelial barrier to prevent molecules from passing between cells. Gap junctions are intercellular channels which allow the passage of small molecules and ions from one cell directly to another. Adherens junctions form apically to desmosomes and organize the actin cytoskeleton (Green et al., 2010; Niessen, 2007). Functionally, desmosomes provide structural support for the entire tissue. Desmosomes' core components consist of cadherins (Desmogleins 1-3 and Desmocollins 1-3), armadillo family proteins (Plakophilins 1-4 and Plakoglobin), and plakins (Desmoplakin) (Delva et al., 2009; Green and Simpson, 2007) (Figure 2.1A). Desmogleins (DSG) and Desmocollins (DSC) are transmembrane proteins which mediate cell-cell adhesion through their extracellular domains and whose cytoplasmic domains serve as an anchoring point for the remaining desmosome components to assemble around at the plasma membrane. Plakophilins (PKP) and Plakoglobin (JUP)

bind to the cadherins and recruit Desmoplakin (DSP) to the plasma membrane (Getsios et al., 2004). PKP/JUP also serve as linkers, bridging DSP to the cadherins. DSP anchors the plasma membrane and the other desmosome components to intermediate filaments. While there are other plakin family members known to associate with desmosomes, DSP is required. It is not yet fully understood how desmosome assembly is regulated or how the components localize to the plasma membrane, but it is important to note that JUP and DSP are both obligatory components and knockout of either DSP or JUP will preclude intact desmosome formation (Garrod and Chidgey, 2008).

While traditionally viewed as being relatively static entities, it has become increasingly appreciated that several of the core desmosome components may also play a role in modulating intracellular signaling, including Wnt signaling (Green and Simpson, 2007; Yang et al., 2012). While down-regulation of several desmosome components has been clearly associated with cancer progression and metastasis, and some desmosome components are even used as diagnostic markers (Boelens et al., 2007; Cui et al., 2012; Papagerakis et al., 2009), the exact mechanism by which their expression and assembly are regulated and how they may modulate intracellular signaling in cancer is not well known. And although decreased expression of desmosome components is a marker indicating EMT has occurred, the mechanism by which YAP may regulate expression of these desmosome components is also unclear. Therefore, we are interested in how YAP/TAZ regulates expression of desmosome components and whether loss of intact desmosomes may contribute to the oncogenic potential of YAP/TAZ. Further, an open question is whether loss of intact desmosomes feeds back to affect Hippo pathway signaling.

2.2 Results

YAP/TAZ activity results in decreased expression of desmosome components

Since increased YAP/TAZ activity has been associated with EMT (Figure S2.1A) and increased cell motility, and one of the early stages of EMT involves the loss of cell junctions, we first used RNA-seq of MCF10A human epithelial cells overexpressing wild-type YAP to determine whether active YAP is sufficient to induce changes in expression of cell junction proteins (Figure 2.1B). Indeed, we found that in the YAP overexpressing cells, expression of nearly all desmosome components was significantly reduced relative to the control cells. This was also correlated with protein expression; in MCF10A cells overexpressing a constitutively-active YAP 5SA mutant, protein levels of cadherin DSG2, as well as obligatory components JUP and DSP, were significantly down-regulated as detected by immunofluorescence (Figure 2.1C). This demonstrates that YAP is able to down-regulate expression of desmosome components both at the RNA level and at the protein level.

We next asked whether this was a phenomenon specific to MCF10A cells, or if it could also be observed in other cell types. Consistently, stimulation of endogenous YAP/TAZ by either serum or Lysophosphatidic acid (LPA) was also sufficient to repress expression levels of DSC2, JUP, and DSP by qPCR in HaCaT keratinocytes, primary cardiomyocytes, and endothelial cells (Figure 2.1D-G). The observation that stimulating YAP/TAZ transiently can affect RNA levels of desmosome components suggests that

desmosomes may be much more dynamically regulated than previously appreciated, and that YAP/TAZ may play a very active role in regulating cell junctions.

YAP/TAZ regulation of desmosome component expression is TEAD1-4 dependent

Because YAP/TAZ do not have their own DNA-binding motifs, they interact with other DNA-binding transcription factors to regulate expression of downstream target genes. The serine 94 residue on YAP is vital for its interaction with TEAD1-4. For example, when this residue is mutated to an alanine, YAP is unable to activate a TEAD1 reporter (Mo et al., 2015). Likewise, when we transfected MCF10A cells with this YAP S94A mutant, it was unable to induce expression of either YAP/TAZ target genes Connective tissue growth factor (CTGF) or Cysteine-rich angiogenic inducer 61 (CYR61) (Figure 2.2A). The wild-type and constitutively-active YAP 5SA mutant both strongly induced CTGF and CYR61, and repressed expression of desmosome components DSC2, JUP, and DSP. However, all of these effects were muted in the YAP S94A mutant, confirming that both induction if YAP/TAZ target genes and down-regulation of desmosome components is largely TEAD1-4 dependent. This is also consistent with what is observed by western blot, that expression of the YAP S94A mutant has no effect on DSC2/3, JUP, or DSP protein levels. Thus, YAP/TAZ regulation of desmosome components is dependent on its interaction with TEAD1-4.

Furthermore, in support of a critical role of TEAD1-4 in regulating expression levels of desmosome components, shRNA knockdown of TEAD1/3/4 in MCF10A cells was sufficient to induce increased levels of DSC2, JUP, and DSP (Figure 2.2C-D). That knockdown of TEAD1-4 alone, under normal growth conditions even in the absence of

any additional stimuli to further activate YAP/TAZ, is able to increase desmosome component expression suggests that TEAD1-4 may play an active role in suppressing their induction or expression.

Gene expression may be regulated at the transcriptional, translational, or at the protein level. We first treated cells with 5-Aza-2'-deoxycytidine (AzaD), a drug which activates methylated genes, to confirm that changes in expression levels of desmosome components are not due to gene silencing or promoter methylation. There were no differences between the YAP overexpressing MCF10A cells and the control cells in response to AzaD treatment (data not shown). Next, to confirm that expression levels of DSC2, JUP, and DSP are primarily regulated via transcription, we treated MCF10A cells with the translation inhibitor cycloheximide (Figure 2.2E). Following treatment, protein levels of each component was dramatically reduced, confirming that changes in protein expression are due to changes in translation and not changes in protein degradation. This was confirmed following treatment with the proteasome-inhibitor MG132, which had no effect on protein levels of any of the components (Figure 2.2F).

YAP/TAZ-TEAD1-4 regulate expression of desmosome components through ZEB1/2

Because TEAD1-4 are transcription factors, one possibility for how they might negatively regulate expression of desmosome components is through inducing expression of a transcription repressor. We compiled a list of known transcription repressors expressed in MCF10A cells and found that repressors ZEB1 and ZEB2 were the most strongly induced in cells overexpressing wild-type YAP (Figure 2.3A). This was

not surprising given the well-established role of ZEB1 as a central mediator and driver of EMT and cancer (Chu et al., 2013; Larsen et al., 2016). This was confirmed by qPCR, as well as the observation that this induction is TEAD1-4 dependent because induction was absent with the YAP S94A mutant.

Moreover, expression of these desmosome components seems primarily regulated through ZEB1/ZEB2, as siRNA knockdown of ZEB1/ZEB2 alone was sufficient to induce increased expression of DSC2, JUP, and DSP, as well as YAP/TAZ target genes CTGF and CYR61 (Figure 2.3C). This is consistent with what was observed in RNA microarray analysis of the Cancer Cell Line Encyclopedia (CCLE), where there were strong negative correlations between ZEB1 expression and DSC2, JUP, and DSP expression (Barretina et al., 2012) (Figure 2.3D). We also identified several putative ZEB1 binding sites in the genomic regions immediately upstream of the transcriptional start sites of each of these genes, suggesting that ZEB1 can directly repress transcription by binding the DNA immediately upstream of these genes.

Loss of desmosomes results in increased migration and YAP/TAZ target gene expression

Skin is subjected to near-constant mechanical stress, and accordingly, keratinocytes form many cell-cell junctions including desmosomes. Desmosome components are highly expressed in human HaCaT cells, making them a model cell line to study the dynamic regulation of desmosomes by YAP/TAZ (Figure 2.4A).

Verteporfin is a chemical inhibitor which prevents YAP/TAZ-TEAD1-4 interaction. Treating HACAT cells with verteporfin was sufficient to suppress expression of

YAP/TAZ target genes CTGF and CYR61, as well as ZEB1 and ZEB2 (Figure 2.4B). Consistently, verteporfin was also sufficient to induce a slight increase in expression of both DSC2 and DSP. These results confirm what we previously identified in the MCF10A cells, namely that YAP/TAZ suppress expression of desmosome components in a TEAD1-4 and ZEB1/ZEB2 dependent manner, and that inhibiting TEAD1-4 can reverse this suppression.

Finally, the last question we were interested in asking was whether loss of desmosomes has any physiological consequences or feedback towards activating YAP/TAZ. Using shRNA, we knocked down obligatory component DSP and found that it also resulted in decreased expression of other desmosome components (Figure 2.4C-D). Without DSP, the cell is unable to form intact desmosomes, although it is not clear how this might affect stability or translation of the remaining components. Knockdown of DSP did not affect expression levels of tight junction components and adherens junction components (data not shown). Moreover, knockdown of DSP was sufficient to induce CTGF and CYR61 expression. Thus, while YAP/TAZ activity results in the down-regulation of desmosome components, the loss of intact desmosomes also confers increased expression of YAP/TAZ target genes. Further work is needed to address whether this is due to changes in YAP/TAZ phosphorylation or localization, or if it may be due to changes in other transcription factors or ZEB1/ZEB2.

To determine whether knockdown of DSP has any physiological consequences, we performed a scratch assay to compare cell migration in either HACAT control cells or DSP knockdown cells. Over a 24-hour period, the DSP knockdown cells migrated faster than the control cells (Figure 2.4E), demonstrating that loss of intact desmosomes

is sufficient confer increased motility. This is consistent with the loss of desmosomes being one of the early stages of EMT. Thus, it is possible that loss of desmosomes is not merely a consequence of ZEB1/ZEB2 induction, but that their downregulation also has some contribution in intracellular signaling and advancing the EMT phenotype.

2.3 Discussion

The Hippo pathway is an important regulator of cell proliferation and apoptosis to maintain tissue homeostasis and control organ size during development and regeneration. Desmosomes are large intercellular junctions which are responsible for maintaining cell-cell adhesion and providing tissues with the strength to withstand mechanical stress. While desmosomes were traditionally thought to be relatively static entities, we found that YAP/TAZ can regulate expression of desmosome components Desmocollin 2 (DSC2), Plakoglobin (JUP), and Desmoplakin (DSP) via transcriptional repression; active YAP/TAZ interact with TEAD1-4 to induce expression of ZEB1 and ZEB2, which repress expression of these components. This highlights a mechanism by which YAP/TAZ play an active role in regulating desmosome expression, and may represent a general pattern in which YAP/TAZ regulate other intercellular junctions as well.

Aberrant YAP/TAZ expression and activity has been correlated with many human cancers and diseases. However, few mutations have been identified in upstream Hippo pathway components, and one of the unanswered questions in the field is how YAP/TAZ become dysregulated during pathogenesis. If other driving mutations or

environmental stresses can induce EMT, our finding that knockdown of DSP can induce expression of YAP/TAZ target genes raises the possibility that down-regulation of desmosome components may feed back to activate YAP/TAZ. This has clear therapeutic implications, as targeting and stabilizing desmosomes may be one way to prevent EMT or aberrant YAP/TAZ activation.

Additionally, even though increased YAP expression has been associated with increased risk of metastasis, the mechanisms by which dysregulated YAP/TAZ can induce cellular changes such as increased motility were not clear. However, the finding that YAP/TAZ activity results in decreased expression of desmosome components and that loss of intact desmosomes itself is sufficient to result in increased motility may yield some new insight into how YAP/TAZ contributes to cancer progression beyond increased cell proliferation and overcoming cell contact inhibition. While these findings are clearly preliminary, and more follow-up detailed mechanistic work is needed, these results offer a new clue as to the physiological consequences of dysregulated YAP/TAZ and remind us how intertwined YAP/TAZ regulation is.

2.4 Experimental procedures

Cell Culture

HEK293A and HaCaT cells were grown in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. MCF10A cells were grown in DMEM/F12 with 5% horse serum (HS), 20 ng/ml EGF, 0.5 mg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 ug/ml Insulin, and 1% penicillin/streptomycin. Cells were also treated with Cycloheximide (75 μg/ml) or MG132 (10 μM) for the indicated times, or starved for 24

hrs before treatment with fresh serum or Lysophosphatidic acid (LPA, 0.5 μM) for 90 minutes.

Western blot

Immunoblots were performed as previously described (Meng et al., 2015). Antibodies used for Desmoplakin (ab16434 and ab109445) and Desmocollin 2/3 (ab69406) are from Abcam, antibodies used for Desmogelin 2 (12631), JUP (2309), CTNNA1 (3240), YAP (14074), TAZ (4883) are from Cell Signaling, and antibodies for ZO1 (sc-10804) and ZO2 (sc-11448) are from Santa Cruz Biotechnologies.

Immunofluorescence

Coverslips were pretreated with 0.0005% Poly-L-ornithine solution (Sigma, P4957) in 12-well plates at 37°C for 15 min and washed with PBS prior to plating cells. Cells were plated 24 hr prior to treatment at medium cell density (1.0×10^5). Cells were fixed in 4% paraformaldehyde for 15 min, followed by permeabilization with 0.1% Triton-X for 5 min and blocking in 3% BSA for 1 hr. Primary antibody was incubated in 3% BSA overnight at 4°C. Secondary antibodies were diluted in 3% BSA and incubated for 1 hr. Slides were mounted with ProLong Gold Antifade Reagent with DAPI.

RNA Extraction, Reverse Transcription, and Real-Time PCR

RNA samples were prepared using the RNeasy Plus mini kit (QIAGEN). Reverse transcription was performed using iScript reverse transcriptase (Bio-Rad). Real-time PCR was performed using KAPA SYBR FAST qPCR master mix (Kapa Biosystems).

RNA Interference

Lentiviral shRNAs were obtained from Sigma Aldrich. ShRNA plasmids together with pMD2.G and psPAX2 were used to produce virus in 293T cells. ON-TARGET plus SMARTpool siRNA were purchased from Dharmacon.

Statistical Analysis

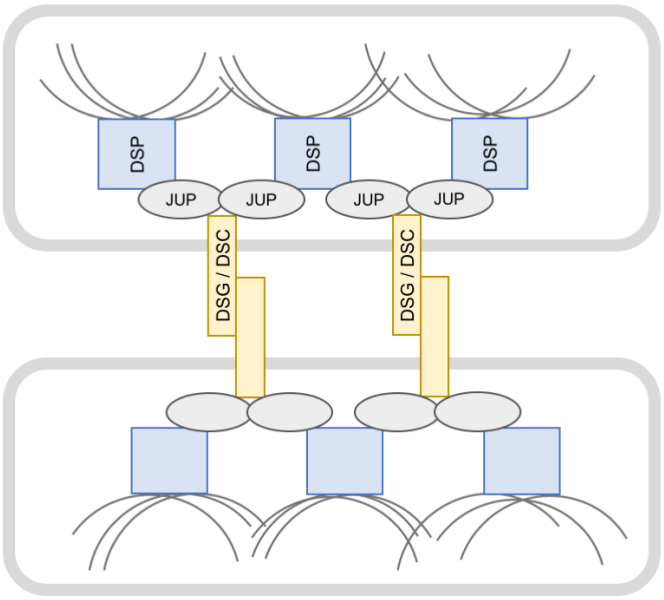
Where indicated, experiments were repeated at least three times and statistical analysis was performed using unpaired t tests. ns, $p > 0.05$; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$.

2.5 Acknowledgments

Chapter 2, in full, is unpublished material; Plouffe, S.W., Guan, K.L. The dissertation author was the primary investigator and author of this material.

Figure 2.1. YAP/TAZ activity results in decreased expression of desmosome components.
A. Schematic of an intact desmosome, including DSP (Desmoplakin) bound to the intermediate filaments, JUP (Plakiglobin), and cadherins DSG/DSC (Desmogleins and Desmocollins). B. Expression levels of desmosome components in MCF10A cells expressing either a control vector or wild-type YAP, as detected by RNA-seq. C. Immunofluorescence showing expression of DSG2, JUP, or DSP (green) and DAPI (DNA stain, blue) in MCF10A cells expressing a constitutively-active YAP 5SA mutant. D-G. Expression detected by qPCR of desmosome components DSC2, JUP, and DSP in epithelial cells, keratinocytes, cardiomyocytes, and endothelial cells following either serum or LPA stimulation to activate endogenous YAP/TAZ.

A



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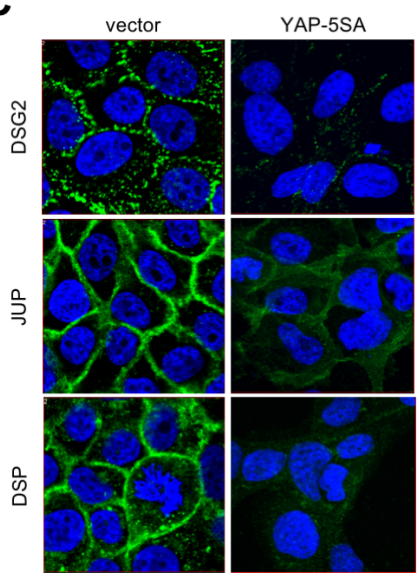
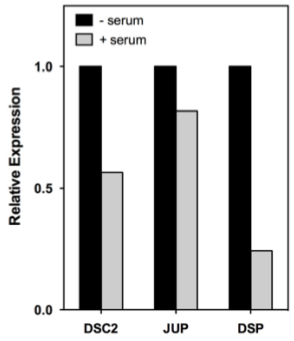


B

	pQCXIH			YAP-wt			Fold Change
PKP3	2053	3772	2884	862	919	1017	0.32
PKP4	299	413	287	210	169	254	0.63
DSC2	2005	3094	2461	48	39	51	0.02
DSC3	2005	3690	2187	256	183	224	0.08
DSG2	348	553	425	437	302	378	0.84
DSG3	906	1399	772	38	40	35	0.04
JUP	2804	4527	3474	3343	2525	2859	0.81
DSP	24663	36762	25249	3895	4245	4490	0.15

D



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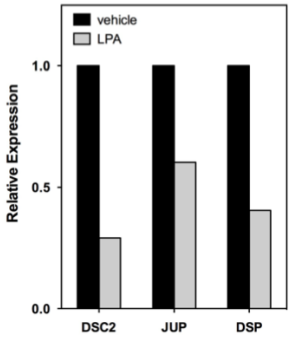

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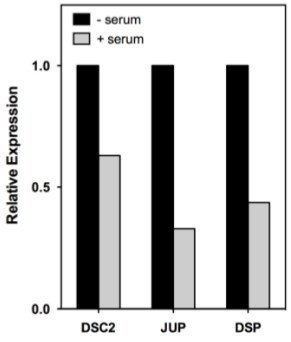

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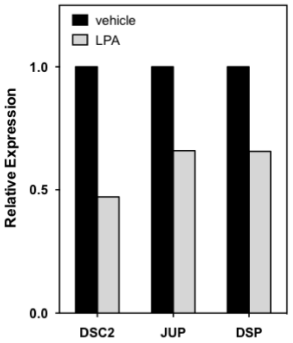


Figure 2.2. YAP/TAZ regulation of desmosome component expression is TEAD1-4 dependent.
A. Expression of desmosome components and YAP/TAZ target genes CTGF and CYR61 in MCF10A cells expressing either wild-type YAP, a constitutively-active mutant 5SA, or a TEAD-binding deficient mutant S94A. B. Western blot showing expression of desmosome components in MCF10A cells. C. qPCR of MCF10A cells following shRNA knockdown of TEAD1/3/4. D. Western blot showing expression of desmosome components following shRNA knockdown of TEAD1/3/4. E-F. Western blot showing expression of desmosome components following treatment with either the translation inhibitor cycloheximide (CHX) or the proteasome inhibitor MG132 for the indicated times. Data represented as mean +/- SD.

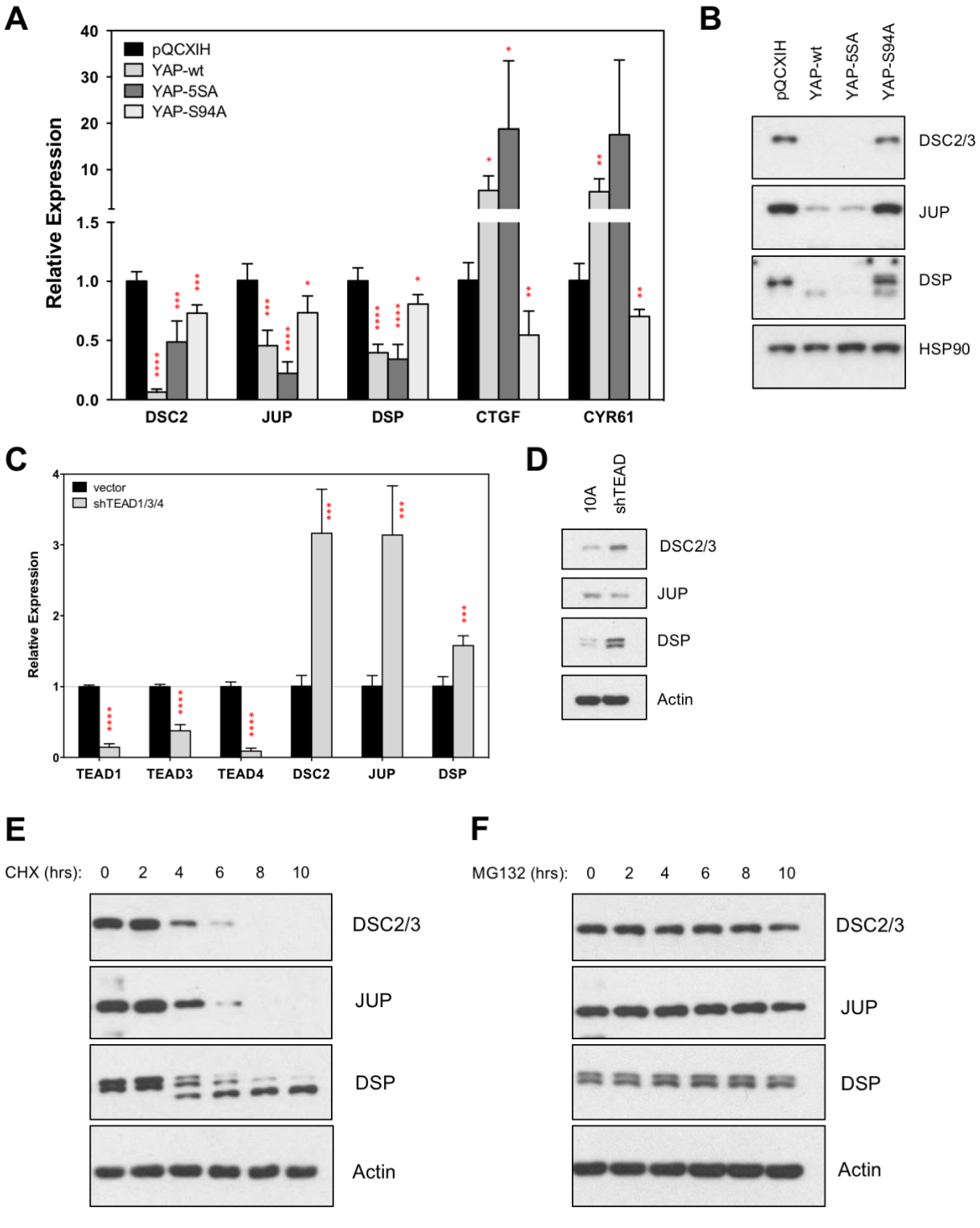
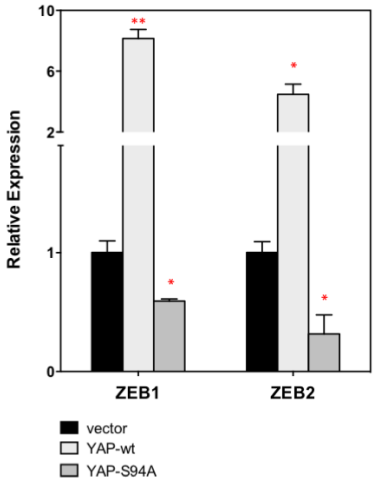

A
Relative Expression
pQCXIH
YAP-wt
YAP-5SA
YAP-S94A
DSC2
JUP
DSP
CTGF
CYR61
B
pQCXIH
YAP-wt
YAP-5SA
YAP-S94A
DSC2/3
JUP
DSP
HSP90
C
Relative Expression
vector
shTEAD1/3/4
TEAD1
TEAD3
TEAD4
DSC2
JUP
DSP
D
10A
shTEAD
DSC2/3
JUP
DSP
Actin
E
CHX (hrs): 0 2 4 6 8 10
DSC2/3
JUP
DSP
Actin
F
MG132 (hrs): 0 2 4 6 8 10
DSC2/3
JUP
DSP
Actin

Figure 2.3. YAP/TAZ-TEAD1-4 regulate expression of desmosome components through ZEB1/2.
A. RNA-seq showing changes in expression of known transcription repressors in MCF10A cells expressing either a control or wild-type YAP. B. qPCR of ZEB1 and ZEB2 in MCF10A cells expressing either a wild-type YAP or a TEAD-binding deficient YAP S94A mutant. C. Expression levels of ZEB1, ZEB2, desmosome components, and YAP/TAZ target genes following ZEB1/ZEB2 siRNA knockdown in MCF10A cells, as detected by qPCR. D. Dot plots showing negative correlation between mRNA expression of desmosome components and ZEB1 expression from 967 subjects. R-values were calculated for each correlation; $P < 0.0001$; Pearson's correlation coefficient. The boxes below represent the genomic regions immediately preceding the transcriptional start sites of DSC2, JUP, and DSP, with ZEB1 consensus binding sites highlighted in red. Data represented as mean +/- SD.

A

	vector			YAP-wt			Fold Change
BCLAF1	1922	2843	1867	2528	2412	2750	1.16
BRCA1	128	198	125	128	132	132	0.87
BRD8	767	1172	724	799	684	770	0.85
CDK7	136	193	119	160	152	141	1.01
CTCF	707	1195	912	956	805	972	0.97
DMTF1	40	73	39	80	75	98	1.66
DR1	327	634	358	627	587	758	1.50
E2F1	32	46	46	46	48	53	1.18
E2F2	30	46	31	13	25	14	0.49
FOXM1	452	792	615	663	481	520	0.90
GTF3C4	71	125	66	65	79	82	0.86
HCFC1	233	401	233	343	315	357	1.17
HIF1A	335	503	370	574	499	580	1.37
HMG20B	603	966	858	1049	629	623	0.95
HMGB2	2429	3554	2898	2122	2450	2386	0.78
ILF2	951	1502	1142	1224	1102	1253	1.00
KDM5B	1619	2773	1755	1687	1456	1680	0.78
KLF6	955	1225	977	1264	1360	1585	1.33
KLF9	31	46	27	67	66	52	1.79
MAPK13	71	127	53	32	33	28	0.37
MNT	632	649	506	520	607	645	0.99
NCOA3	1377	1942	1118	1071	1074	1400	0.80
NFE2L2	1942	2765	1990	1111	1144	1326	0.53
NFIC	391	769	481	913	840	1011	1.68
NR3C1	1455	2571	1223	1059	920	1183	0.60
PCNA	98	179	105	82	46	71	0.52
PHTF2	161	189	126	128	127	130	0.81
PKNOX1	150	299	216	154	138	155	0.67
PTTG1	1916	2410	2561	652	580	553	0.26
RBPJ	451	675	557	1052	899	976	1.74
SCML1	75	95	66	119	89	120	1.40
SP1	1295	1894	1562	1127	936	998	0.64
SRF	451	726	568	528	504	500	0.88
STAT1	2201	3197	2427	1625	1439	1529	0.59
STAT5B	180	217	189	418	307	361	1.85
TCERG1	255	397	266	234	231	212	0.74
TFAP2A	2748	4239	3048	2338	2640	2879	0.78
UHRF1	21	33	26	28	23	30	1.01
ZEB1	41	61	48	311	254	283	5.67
ZEB2	7	28	14	78	35	34	2.99
ZNF207	1571	2254	1930	1816	1432	1587	0.84
ZNF24	317	498	359	435	380	424	1.05
ZNF281	165	231	209	258	178	216	1.08

B



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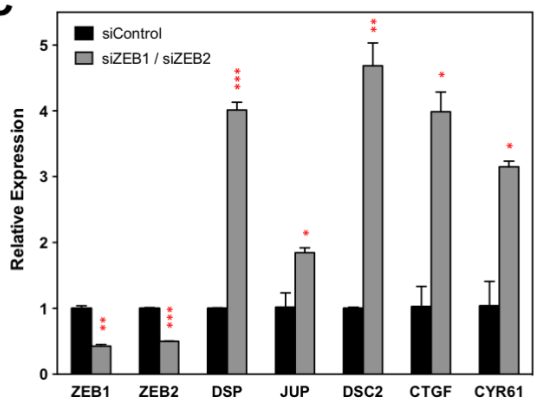

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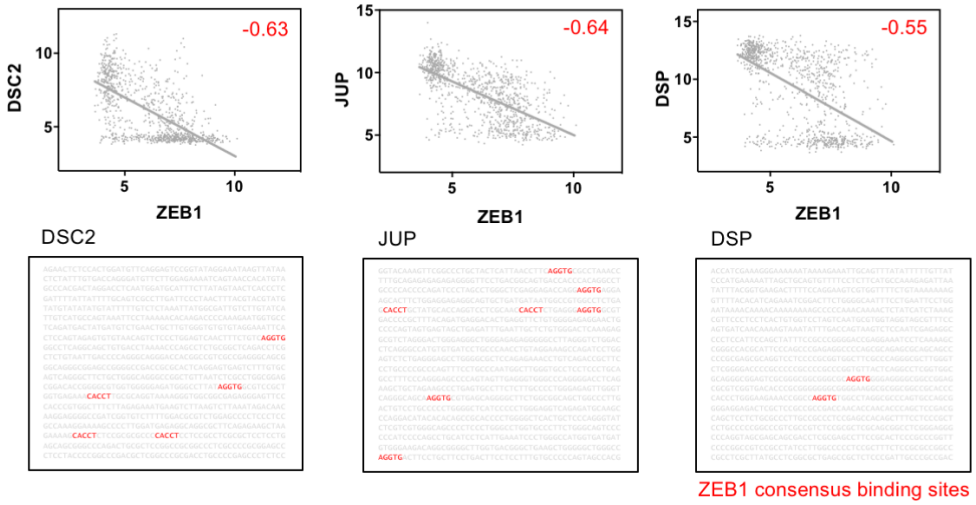
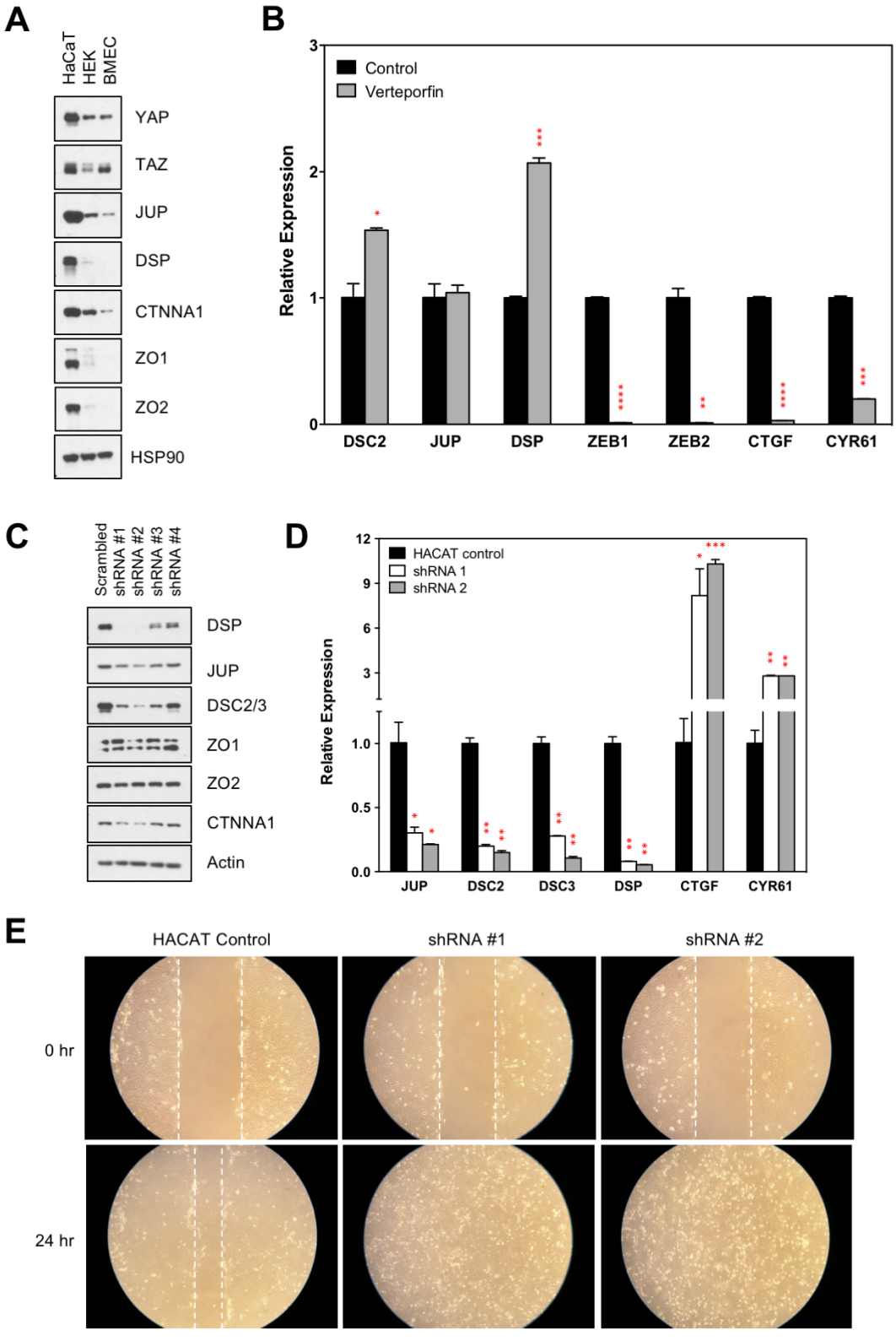


Figure 2.4. Loss of desmosomes results in increased migration and YAP/TAZ target gene expression.
A. Western blot showing expression of intercellular junction components in HACAT, HEK293A, and BMEC cells. B. HACAT cells were treated with Verteporfin (1 ug/ml) and expression levels of indicated genes were detected by qPCR. C. Western blot showing expression of desmosome components in HACAT cells following infection with 4 different shRNA for DSP. D. RNA expression of desmosome components and YAP/TAZ target genes following shRNA knockdown of DSP in HACAT cells. E. Scratch assay of either control HACAT cells or HACAT cells following shRNA knockdown of DSP. Data represented as mean +/- SD.


A
HaCaT
HEK
BMEC
YAP
TAZ
JUP
DSP
CTNNA1
ZO1
ZO2
HSP90
B
Control
Verteporfin
Relative Expression
DSC2
JUP
DSP
ZEB1
ZEB2
CTGF
CYR61
C
Scrambled
shRNA #1
shRNA #2
shRNA #3
shRNA #4
DSP
JUP
DSC2/3
ZO1
ZO2
CTNNA1
Actin
D
HACAT control
shRNA 1
shRNA 2
Relative Expression
JUP
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DSP
CTGF
CYR61
E
HACAT Control
shRNA #1
shRNA #2
0 hr
24 hr

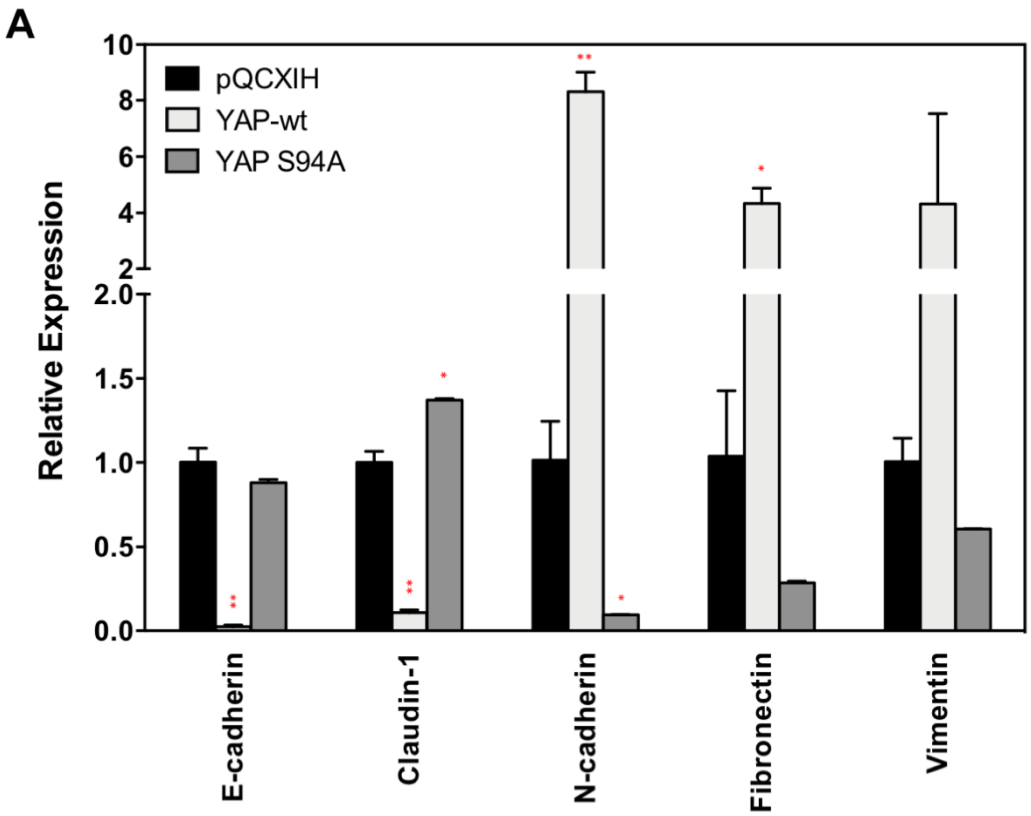

Figure S2.1 YAP overexpression suppresses expression of epithelial markers and induces expression of mesenchymal markers.
A. qPCR of MCF10A cells expressing either wild-type YAP or a TEAD-binding deficient YAP S94A mutant. E-cadherin and Claudin-1 are epithelial markers, while N-cadherin, Fibronectin, and Vimentin are mesenchymal markers.

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Chapter 3: Characterization of Hippo pathway components by gene inactivation

3.1 Introduction

The Hippo pathway is a well-established regulator of tissue homeostasis (Yu et al., 2015b). The mammalian Hippo pathway consists of a kinase cascade of Mammalian STE20-like 1/2 (MST1/2) and Large Tumor Suppressor 1/2 (LATS1/2), which inhibit the primary effectors of the Hippo pathway, Yes Associated Protein (YAP) and WW Domain-containing Transcription Factor (TAZ). When the Hippo pathway is activated, MST1/2 phosphorylates its adaptor protein Salvador 1 (SAV1), which facilitates MST1/2-LATS1/2 interaction (Callus et al., 2006; Tapon et al., 2002). MST1/2 then phosphorylates LATS1/2 at its hydrophobic motif (HM: threonines 1079 on LATS1 and 1041 on LATS2), which promotes LATS1/2 auto-phosphorylation at its activation loop. MST1/2 also phosphorylates MOB Kinase Activator 1A/B (MOB1A/B) at threonine 35, enabling MOB1A/B to bind the auto-inhibitory region of LATS1/2 and promote full LATS1/2 activation (Chan et al., 2005; Praskova et al., 2008). Once activated, LATS1/2 can directly phosphorylate YAP and TAZ.

LATS1/2-dependent phosphorylation of YAP serine 127 results in YAP binding to 14-3-3, sequestration in the cytoplasm, ubiquitination, and degradation (Dong et al., 2007; Liu et al., 2010; Zhao et al., 2010b; Zhao et al., 2007). LATS1/2 also regulate TAZ protein localization and stability in a similar manner, although phosphorylation of TAZ occurs on different residues and TAZ is more unstable due to an additional phosphodegron. Dephosphorylated YAP/TAZ translocate to the nucleus where they act as transcriptional co-activators, interacting with transcription factors to induce

expression of genes regulating cell proliferation, apoptosis, and differentiation (Zhao et al., 2008).

Disrupting the Hippo pathway results in the loss of tissue homeostasis. For example, deleting Hippo or Warts (the *Drosophila* homologs of MST1/2 and LATS1/2, respectively) is sufficient to cause aberrant Yorki (the *Drosophila* homolog of YAP/TAZ) activity and uncontrolled growth in both eye and wing (Huang et al., 2005; Pan, 2010). Similarly, conditionally deleting MST1/2 or LATS1/2 in the mouse liver results in YAP/TAZ accumulation and massive hepatomegaly and tumors (Chen et al., 2015; Yu et al., 2015a).

Not surprisingly, dysregulation of the Hippo pathway has been implicated in many human diseases (Plouffe et al., 2015). YAP amplification and increased YAP/TAZ nuclear localization have been correlated with an increased risk of metastasis and decreased survival in lung, colorectal, and breast cancers, to name a few (Wang et al., 2012; Wang et al., 2010; Wierzbicki et al., 2013). However, the mechanisms by which the Hippo pathway becomes dysregulated are not fully understood; few mutations in core Hippo pathway components have been identified in human cancers (Harvey et al., 2013).

Therefore, much work has focused on identifying upstream regulators of the Hippo pathway which may contribute to aberrant YAP/TAZ activity in disease. Several studies recently identified the Mitogen-Activated Protein Kinase kinase kinase kinase (MAP4K) family as direct activators of LATS1/2, acting in parallel to MST1/2 (Li et al., 2014a; Meng et al., 2015; Zheng et al., 2015). Other work has greatly expanded the Hippo interactome to include Ras Association Domain Family Member 1A (RASSF1A),

Tao Kinases 1-3 (TAOK1/2/3), AMPK (PRKKA1/PRKKA2), Protein Kinase A (PRKACA/PRKCB), Ras Homology Family Member A (RHOA), Neurofibromin 2 (NF2), Angiomotin (AMOT), Catenin Alpha 1 (CTNNA1), and Ajuba Lim Protein (AJUBA) (Figure 3.1A). While the functions of these components in regulating the Hippo pathway have been well studied by either knockdown or knockout, most studies have focused only on individual components, emphasizing only the importance of the component of interest to that particular study. Thus, it is not always clear how the contribution of each component compares relative to others, nor which are the most physiologically relevant in regulating YAP/TAZ. To promote a fuller understanding of the Hippo pathway, we created knockout cell lines for each of these components in HEK293A cells using CRISPR/Cas9 and compared their relative contributions in regulating YAP/TAZ phosphorylation and localization. Our study provides an overarching view of known Hippo pathway components in YAP/TAZ regulation in response to a wide range of physiological signals and identifies components which, when deleted, are sufficient to cause significant YAP/TAZ dysregulation.

3.2 Results

Role of Hippo pathway components in regulating YAP/TAZ phosphorylation in response to serum starvation

First, to demonstrate that this approach is sufficient to identify the most critical regulators of YAP/TAZ, we generated knockout cell lines of the Hippo pathway core components (Figures 3.1A-1B, S3.1, S3.7, and Table S3.1). One of the most robust signals to regulate YAP/TAZ activity is serum; lysophosphatidic acid (LPA) and

sphingosine 1-phosphate in serum act through G-protein coupled receptors (GPCRs) to inactivate the Hippo pathway (Miller et al., 2012; Yu et al., 2012). When the Hippo pathway is inactive, YAP/TAZ are dephosphorylated and translocate to the nucleus to induce transcription. During serum starvation, the Hippo pathway is activated and YAP/TAZ are phosphorylated and sequestered in the cytoplasm. YAP/TAZ phosphorylation can be detected by phosphorylation-specific antibodies and by mobility shift (Figure 3.1C).

As previously reported, deleting either MST1/2 or the MAP4K family alone did not significantly disrupt YAP/TAZ regulation, but deleting both MST1/2 and the MAP4Ks compromised YAP/TAZ phosphorylation in response to serum starvation (Figure 3.1C and S2.2). Deleting LATS1/2 blocked nearly all YAP/TAZ phosphorylation, and deleting MOB1A/B severely compromised YAP/TAZ phosphorylation as well. The effect of deleting MOB1A/B was more dramatic than deleting MST1/2 but less than LATS1/2, as trace levels of phosphorylated YAP (S127) were still present in the MOB1A/B KO cells (Figure 3.1C). These results demonstrate that LATS1/2 has some intrinsic activity to phosphorylate YAP/TAZ independent of MOB1A/B, although MOB1A/B is necessary to promote full LATS1/2 activation (Chan et al., 2005; Praskova et al., 2008). This is consistent with reported animal studies which found that deletion of MOB1A and a homozygous-null mutation in MOB1B resulted in increased YAP activity and hyper-proliferation (Nishio et al., 2012). In the absence of MOB1A/B, the cell is unable to fully activate LATS1/2 to inhibit YAP/TAZ.

While the YAP mobility shift by phos-tag is consistent with YAP (S127) phosphorylation, the phos-tag also provides more quantitative information for all YAP

phosphorylation sites (Figure 3.1C). For this reason we used phos-tag as the primary readout to compare overall YAP phosphorylation for subsequent experiments.

To better compare the contribution of each component, we performed a time course of serum starvation (Figure 3.2 and S3.3). Upon starvation, YAP phosphorylation occurred rapidly after 15-30 minutes, with no observed intermediate bands of partially-phosphorylated YAP in the wild-type cells. This suggests that virtually all YAP sites undergo phosphorylation in all cells synchronously, revealing an extremely tight regulation of YAP phosphorylation. TAZ phosphorylation also occurred after 15-30 minutes, although phosphorylation of TAZ was less efficient (Figure 3.2A); TAZ was only partially phosphorylated after 90 minutes of starvation, indicating that endogenous YAP is more efficiently phosphorylated than TAZ. TAZ phosphorylation was also confirmed by phos-tag (Figure S3.2C-E).

Deleting SAV1 or MST1/2 resulted in a significant delay of YAP/TAZ phosphorylation compared to the wild type cells, although interestingly, the SAV1 KO cells showed a greater delay in YAP/TAZ phosphorylation than the MST1/2 KO cells, suggesting that SAV1 may have other functions beyond interacting with MST1/2 (Figure 3.2A and S3.3A). In addition, even after 90 minutes of starvation, YAP was not fully phosphorylated in either cell line. We next compared the effect of deleting LATS1 or LATS2. YAP phosphorylation was modestly compromised in the LATS1 KO cells but unaffected in the LATS2 KO cells. However, deleting both LATS1/2 abolished YAP/TAZ phosphorylation. This response could be rescued by re-expressing LATS1/2 (Moroishi et al., 2015). As previously discussed, the MST1/2-MAP4K1/2/3/4/6/7 8KO (MST-MAP4K 8KO), MOB1A/B KO, and LATS1/2 KO cells all exhibited severe defects in

starvation-induced YAP/TAZ phosphorylation; without these core components, the cell was unable to inactivate YAP/TAZ. Together, the above indications confirm the utility of this approach to compare and identify Hippo pathway components which are the most physiologically important in regulating YAP/TAZ.

TAOK has been reported to phosphorylate and activate MST1/2 (Boggiano et al., 2011; Poon et al., 2011). We attempted deleting TAOK1/2/3. Deletion of TAOK1/2 was confirmed by immunoblot and sequencing (Figure S3.1A, S3.1W-X). However, we were unable to verify complete deletion of TAOK3 due to the relatively low quality of the TAOK3 antibody (Figure S3.1J). Sequencing the sgRNA target site was also hindered due to multiple genomic copies of TAOK3 in HEK293A cells. Nevertheless, the TAOK1/2/3 KO cell lines showed a significant delay in YAP/TAZ phosphorylation upon serum starvation, similar to that of the MST1/2 KO cells (Figure 3.2B and S3.3B). Deleting TAOK1/2/3 on top of the MST1/2-MAP4K4/6/7 5KO cells resulted in complete YAP/TAZ dephosphorylation, while the MST1/2-MAP4K4/6/7 5KO cells retained some YAP phosphorylation. This data suggests that TAOK1/2/3 may have additional activity independent of MST1/2 to activate LATS1/2, which will be further examined in Figure 3.6.

The CTNNA1 and TAZ KO cells showed no difference in YAP phosphorylation relative to the wild-type cells. The AJUBA, AMOT, and RASSF1A KO cells were more sensitive to serum starvation (Figure 3.2C and S3.3C). The AJUBA KO cells phosphorylated YAP within 10 minutes of starvation, and the AMOT and RASSF1A KO cells showed YAP phosphorylation within 15 minutes of starvation. AJUBA is localized at adherens junctions and interacts with SAV1 and LATS1/2 to promote YAP/TAZ

phosphorylation, so its role in response to serum starvation is not clear (Das Thakur et al., 2010). The contribution of AMOT to the Hippo pathway is also not well understood. Although AMOT can induce LATS2-mediated phosphorylation of YAP and sequester YAP/TAZ to tight junctions (Chan et al., 2011; Paramasivam et al., 2011; Wang et al., 2011; Zhao et al., 2010a), AMOT is also reported to activate YAP by binding YAP in the cytoplasm to prevent LATS1/2-mediated phosphorylation and to promote transcription (Yi et al., 2013). Moreover, AMOT binds to and activates NF2 (Li et al., 2015). Here, in response to serum starvation, the AMOT KO cells were slightly more sensitive but overall did not show much difference from the wild-type cells. However, Angiomotin family members AMOTL1 and AMOT2 remained intact in the AMOT KO cells. Because they are closely related, AMOTL1 and AMOTL2 may be functionally redundant with AMOT, which may explain why we did not observe much change in the AMOT KO cells (Chan et al., 2011; Zhao et al., 2010a). RASSF1A acts upstream of MST1/2, but the observation that YAP/TAZ were highly phosphorylated in the RASSF1A KO cells indicates that the RASSF1A contribution to the Hippo pathway in response to serum starvation is not indispensable.

Surprisingly, the AMPK and PKA KO cells had higher basal levels of phosphorylated YAP even in the presence of serum (Figure 3.2C and S3.3C). AMPK and PKA are known to promote YAP phosphorylation. Thus, it was unexpected that deleting AMPK or PKA would result in higher basal levels of phosphorylated YAP, although this may reflect a cellular adaptation.

Of all the components tested, NF2 and RHOA showed the most significant, though contrasting, dysregulation of YAP/TAZ. In the NF2 KO cells, YAP/TAZ remained

dephosphorylated following serum starvation, while YAP/TAZ were fully phosphorylated in the RHOA KO cells even in the presence of serum (Figure 3.2D and S3.3D). These data demonstrate that NF2 and RHOA play dominant roles in LATS1/2 regulation, NF2 in positively regulating LATS1/2 activity and RHOA in negatively regulating LATS1/2 activity. These will be further examined in Figures 3.5 and 3.7.

Role of Hippo pathway components in regulating YAP/TAZ localization and transcriptional activity in response to serum starvation

Phosphorylated YAP/TAZ are sequestered in the cytoplasm by binding to 14-3-3, while dephosphorylated YAP/TAZ translocate to the nucleus to induce transcription. YAP/TAZ protein localization was examined by immunofluorescence and found to be consistent with the observed YAP/TAZ phosphorylation status. For all cell lines shown, in the presence of serum, YAP/TAZ were nuclear (Figure 3.3A and S3.4A). Following prolonged serum starvation, YAP/TAZ were primarily cytoplasmic in the wild-type, SAV1 KO, MST1/2 KO, and MAP4K4/6/7 KO cells. Weak YAP/TAZ staining following starvation was due to YAP/TAZ degradation and diffuse cytoplasmic localization. However, in the MST-MAP4K 8KO, MOB1A/B KO, and LATS1/2 KO cells, YAP/TAZ remained nuclear even following prolonged serum starvation. These data further demonstrate that, without these core components, the cell is unable to sequester and inactivate YAP/TAZ in the cytoplasm.

Dysregulation of YAP/TAZ phosphorylation and localization is sufficient to drive changes in YAP/TAZ transcriptional activity. Cysteine Rich Angiogenic Inducer 61 (*CYR61*) and Connective Tissue Growth Factor (*CTGF*) are two well-established target

genes induced by YAP/TAZ (Zhao et al., 2008). Even in the presence of serum, MOB1A/B KO and LATS1/2 KO cells had higher CYR61 and CTGF expression than the wild-type cells (Figure 3.3B). This was consistent with previous observations that, even in the presence of serum, YAP/TAZ are not completely dephosphorylated in the wild-type cells (Figure 3.1C). This difference became more pronounced following serum starvation (Figure 3.3C). Following starvation, CYR61 and CTGF expression were strongly reduced in the wild-type cells as YAP/TAZ were sequestered in the cytoplasm. However, YAP/TAZ remained nuclear in the MOB1A/B and LATS1/2 KO cells and CYR61 and CTGF were not repressed. The low level of MOB1A/B-independent LATS1/2 activity towards YAP/TAZ may explain why expression of CYR61 and CTGF expression were greater in the LATS1/2 KO cells than the MOB1A/B KO cells.

We next examined the effect of dysregulated YAP/TAZ on cellular metabolism. Rapidly growing cells metabolize glucose in the culture medium and release lactic acid via glycolysis, which lowers the medium's pH. To compare the relative rates of metabolism across the different cell lines, cells were given fresh media for 6 hours, after which we measured the culture medium's remaining glucose levels and pH (Figure 3.3D and 3.3E). YAP/TAZ KO cells had a lower metabolic rate than the wild-type cells, as indicated by their culture medium's higher remaining glucose levels and pH. These results support that YAP/TAZ activity stimulates glucose uptake and glycolysis, consistent with the cell growth-promoting activity of YAP/TAZ. The MST1/2 KO cells had lower glucose levels and pH than the wild-type cells, consistent with a negative role for MST1/2 in YAP/TAZ regulation. The MOB1A/B and LATS1/2 KO cells had even lower glucose levels and pH, which is consistent with their increased YAP/TAZ transcriptional

activity. Together, these data indicate that aberrant Hippo pathway activity is sufficient to cause metabolic changes in the cell.

Cells were additionally treated with Latrunculin B to disrupt the actin cytoskeleton and Forskolin/3-isobutyl-1-methylxanthine (F/IBMX) to activate PKA. Consistent with previous studies, Latrunculin B and F/IBMX activate the Hippo pathway and induce YAP phosphorylation (Figure S3.5A and S3.5B). Even under these conditions, YAP/TAZ remained dephosphorylated in the MST-MAP4K 8KO, MOB1A/B KO, and LATS1/2 KO cells.

Energy starvation is also known to induce YAP/TAZ phosphorylation. We used 2-Deoxy-D-glucose (2-DG), which inhibits glucose metabolism, to induce cellular energy stress. Deletion of SAV1, MST1/2, or MAP4K4/6/7 did not abolish 2-DG-induced YAP/TAZ phosphorylation (Figure S3.5C). Notably, in these cells, YAP/TAZ phosphorylation peaked after 60 minutes of 2-DG treatment before declining, while no such decline was evident in the wild-type cells. This indicates that the dynamics of the cellular energy stress response are altered in these KO cells. In the MST1/2-MAP4K4/6/7 KO, MST-MAP4K 8KO, MOB1A/B KO, and LATS1/2 KO cells, 2-DG induced partial but not full YAP phosphorylation. This slight upward shift was likely due to LATS1/2-independent YAP phosphorylation by AMPK (Mo et al., 2015; Wang et al., 2015). However, when AMPK was deleted, YAP/TAZ were still fully phosphorylated in response to energy stress, suggesting an AMPK-independent mechanism of LATS1/2 activation. Deletion of PKA partially blocked the effect of 2-DG on YAP phosphorylation. Although YAP was already highly phosphorylated in the RHOA KO cells, phosphorylation of TAZ was still clearly induced in the RHOA KO cells, indicating that

RHOA deletion does not block the energy stress response. Collectively, our data indicate that many upstream components may play a role in regulating YAP/TAZ in response to cellular energy stress.

Different mechanisms may regulate YAP/TAZ in response to cell-cell contact than serum starvation

The Hippo pathway is also strongly regulated by cell-cell contact. In response to cell-cell contact, the Hippo pathway is activated and YAP/TAZ are phosphorylated and inactivated to prevent further growth and proliferation. To test the mechanisms of YAP regulation in response to cell-cell contact, cells were plated at three densities and YAP phosphorylation status was examined (Figure 3.4A). When comparing the core Hippo pathway component knockout cells, high density induced YAP/TAZ phosphorylation in all except the LATS1/2 KO cells (Figure 3.4B and 3.4C). Intriguingly, density-induced YAP phosphorylation was significantly compromised in the MST1/2-MAP4K4/6/7 KO, MST-MAP4K 8KO, and MOB1A/B KO cells, although these cells were still clearly able to phosphorylate YAP at high density. YAP/TAZ protein localization was also consistent with these observations (Figure 3.4D and S3.4B). At low density, YAP/TAZ were nuclear in all cells. At medium density in the wild-type, SAV1 KO, MST1/2 KO, MAP4K4/6/7 KO, and MST-MAP4K 8KO cells, YAP/TAZ were cytoplasmic. However, YAP/TAZ showed partial and complete nuclear localization in the MOB1A/B KO and LATS1/2 KO cells, respectively.

From the above data, it is clear that the signal transduction regulating YAP/TAZ in response to cell-cell contact is either different or more severe than those in response

to serum starvation. It is important to note that cells grown at high density may also experience limited nutrients, although we already observed that even overnight starvation is not sufficient to induce YAP phosphorylation in the MST-MAP4K 8KO or MOB1A/B KO cells (Figure 3.1C). However, LATS1/2 are the primary kinases for YAP/TAZ in response to cell-cell contact because deleting LATS1/2 abolished YAP/TAZ phosphorylation and cytoplasmic localization, even at high density.

Besides the LATS1/2 KO cells, the only cell line which showed dramatic disruption of YAP/TAZ phosphorylation was the MST1/2-MAP4K4/6/7-TAOK1/2/3 8KO (MST-MAP4K-TAOK 8KO) cells (Figure 3.4B). Although trace levels of phosphorylated YAP were present, the majority of YAP remained dephosphorylated even at high density. This was a striking difference when compared to both the TAOK1/2/3 KO and MST1/2-MAP4K4/6/7 KO lines. These data again indicate that TAOK1/2/3 must have additional activity apart from acting upstream of MST1/2 or the MAP4K family.

Many components have been implicated in regulating LATS1/2 kinase activity or YAP/TAZ localization in response to cell-cell contact, including AJUBA, CTNNA1, and AMOT. However, each of these KO cells showed strong YAP phosphorylation at both medium and high densities (Figure 3.4B). Notably, the NF2 KO cells showed reduced phosphorylation, particularly at medium density, in support of previous reports that NF2 is an important mediator for cell-cell contact-induced YAP/TAZ phosphorylation (Yin et al., 2013). Nevertheless, our data clearly show NF2-independent activation of the Hippo pathway as YAP was still highly phosphorylated at high density in the NF2 KO cells. Deleting some cell junction-associated proteins resulted in altered cellular morphology, which may also influence the Hippo pathway. CTNNA1 is required for adherens

junctions, and the CTNNA1 KO cells appeared incapable of forming adherens junctions (Figure 3.4E). Nevertheless, the CTNNA1 KO cells remained sensitive to cell-cell contact, which could be due to other cell-cell junctions or changes in cell shape at higher densities.

NF2 knockout cells have hyper-activated YAP/TAZ

NF2 is a well-established tumor suppressor and is often mutated in neurofibromatosis and mesothelioma. In patients with Neurofibromatosis type 2, inactivating mutations in NF2 have been correlated with increased YAP expression and nuclear localization (Schulz et al., 2014; Striedinger et al., 2008). As discussed previously, deleting NF2 severely compromised YAP/TAZ phosphorylation in response to serum starvation (Figure 3.2D). Accordingly, serum starvation also induced YAP/TAZ cytoplasmic localization in wild-type cells but not the NF2 KO cells (Figure 3.5A and S3.4C). This dysregulation of YAP/TAZ phosphorylation and localization was consistent with the increased expression of YAP/TAZ target genes CYR61 and CTGF in the NF2 KO cells (Figure 3.5B and 3.5C). The NF2 KO cells were also resistant to YAP/TAZ cytoplasmic localization when grown at increased density in the presence of serum. However, the combination of increased density and prolonged serum starvation was able to induce YAP/TAZ cytoplasmic localization in the NF2 KO cells (Figure 3.5D and S3.4C). Thus, while YAP/TAZ seem largely resistant to regulation when NF2 is deleted, in response to stronger signals (such as the combination of increased density and prolonged serum starvation), the cell is able to override loss of NF2 and inactivate YAP/TAZ. Re-expressing NF2 was sufficient to rescue YAP phosphorylation (Moroishi

et al., 2015).

NF2 has been reported to promote LATS1/2 activation by inhibiting LATS1/2 ubiquitination and degradation in the nucleus (Li et al., 2014b). We found that LATS1 protein levels were relatively unchanged in the NF2 KO cells compared to the wild-type cells, although LATS2 expression was decreased slightly (Figure 3.5E). Nor did we observe any difference in LATS1/2 protein stability following treatment with the translation-inhibitor cycloheximide (Figure 3.5F). These data suggest that, at least in HEK293A cells, NF2 affects LATS1/2 localization or kinase activity but not protein stability. MST1/2 are direct kinases for MOB1A/B and LATS1/2, and deleting MST1/2 was sufficient to disrupt all MOB1A/B phosphorylation. However, deleting NF2 dramatically reduced LATS1/2 (HM) phosphorylation but had no effect on MOB1A/B phosphorylation (Figure 3.5E), suggesting that NF2 KO does not affect MST1/2 kinase activity but affects MST1/2-dependent phosphorylation of LATS1/2 by potentially altering LATS1/2 localization.

In addition to increased transcription of YAP/TAZ target genes, deleting NF2 confers a growth advantage as well; NF2 KO cells proliferated at a faster rate than the wild-type cells (Figure 3.5G). Nevertheless, in addition to combined density and prolonged starvation, there remained some conditions in which YAP/TAZ could be weakly regulated in the NF2 KO cells. Actin disruption by Latrunculin B was able to induce weak YAP phosphorylation (Figure 3.5H), and the NF2 KO cells showed altered response to energy stress. Although 2-DG treatment induced some YAP/TAZ phosphorylation after 60 minutes, the YAP/TAZ phosphorylation in the NF2 KO cells appeared less robust and recovered quicker than the wild-type cells, as significant YAP

dephosphorylation was present after 90 minutes of 2-DG treatment (Figure 3.5I). Furthermore, phosphorylation of LATS1/2 (HM) and YAP (S127) remained severely compromised in the NF2 KO cells, supporting a critical role for NF2 in Hippo pathway regulation.

TAOK1/3 act upstream of MST1/2 and MAP4K to phosphorylate and activate LATS1/2

In *Drosophila*, Tao kinase 1 (Tao-1) directly phosphorylates Hippo, and the mammalian TAOK1 can phosphorylate and activate MST2 in vitro (Boggiano et al., 2011; Poon et al., 2011). Deleting TAOK1/2/3 had a slight effect on YAP/TAZ phosphorylation in response to serum starvation, as YAP/TAZ phosphorylation was delayed and less robust than in the wild-type cells (Figure 3.2B). However, as discussed previously, deleting TAOK1/2/3 in the MST1/2-MAP4K4/6/7 5KO cells almost completely abolished YAP/TAZ phosphorylation in response to serum starvation and cell-cell contact (Figure 3.2B and 3.4B). These data suggest that TAOK1/2/3 may also act in parallel to MST1/2 and MAP4K4/6/7 to activate LATS1/2 and induce YAP/TAZ phosphorylation. Consistently, overexpressing TAOK1 was sufficient to induce LATS1/2 (HM) phosphorylation (Figure 3.6A). In addition, TAOK1 and TAOK3 directly phosphorylated LATS1/2 (HM) in an in vitro kinase assay (Figure 3.6B). Therefore, TAOK1/3 may act both upstream of and in parallel to MST1/2 and MAP4K to phosphorylate and activate LATS1/2. To test this, we overexpressed TAOK1 in various knockout cell lines. Overexpressing TAOK1 was sufficient to induce YAP phosphorylation even in the MST1/2-MAP4K4/6/7 5KO and MST-MAP4K 8KO cells, but

not the MOB1A/B KO or LATS1/2 KO cells, thereby confirming that MST1/2 and the MAP4K family are not required for TAOK1 to induce YAP phosphorylation (Figure 3.6C). We further examined the effect of TAOK on YAP/TAZ localization. Density-induced YAP/TAZ cytoplasmic localization was observed in the MST1/2-MAP4K4/6/7 5KO and TAOK1/2/3 KO cells, but not in the MST-MAP4K-TAOK 8KO cells (Figure 3.6E and S3.4D). These data further support a model that TAOK1/3 can inhibit YAP/TAZ independent of MST1/2 and the MAP4Ks, and can directly phosphorylate and activate LATS1/2.

A prominent role for RHOA in YAP/TAZ activation

RHOA plays an important role in transducing signals from GPCRs to regulate F-actin. Treating cells with the RHO inhibitor C3 exoenzyme (C3) strongly induced LATS1/2 (HM) and YAP (S127) phosphorylation (Figure 3.7A). C3 also activated, though weakly, LATS1/2 and induced YAP phosphorylation in the MST-MAP4K 8KO, MOB1A/B KO, and MST-MAP4K-TAOK 8KO cells. Although RHOA, RHOB, and RHOC all share significant homology, they have distinct localizations and functions, with RHOA primarily acting to regulate the actin cytoskeleton. By RNA-seq, the expression of RHOB and RHOC in HEK293A cells are significantly lower than that of RHOA (Sultan et al., 2008) (Figure S3.1Z). Therefore, we hypothesized that the effect of C3 on YAP phosphorylation was primarily through RHOA. Deleting RHOA altered the cellular morphology, and the RHOA KO cells appeared to have increased filopodia (Figure 3.7B). Furthermore, deleting RHOA resulted in hyper-phosphorylated LATS1/2 and YAP/TAZ (Figure 3.7A). Accordingly, CYR61 and CTGF expression were also

decreased in the RHOA KO cells (Figure 3.7C).

Neither serum nor LPA could induce YAP/TAZ dephosphorylation in the RHOA KO cells (Figure 3.2D, 3.7D, and S3.6A). These data support a critical role for RHOA in mediating LPA-induced GPCR signaling to the Hippo pathway. Recently, we reported that activation of conventional PKC by 12-O-tetradecanoylphorbol-13-acetate (TPA) could induce YAP/TAZ activation (Figure S3.6B) (Gong et al., 2015). Interestingly, TPA failed to induce YAP dephosphorylation, but TAZ was clearly dephosphorylated in the RHOA KO cells in response to TPA stimulation (Figure 3.7D). TPA, but not serum or LPA, also induced significant YAP/TAZ nuclear localization (Figure 3.7E and S3.4E). This observation raises the possibility that PKC acts partly via RHOA to regulate YAP/TAZ activity.

3.3 Discussion

The Hippo pathway plays an important role in maintaining tissue homeostasis by regulating cell proliferation, apoptosis, and differentiation. Although dysregulation of the Hippo pathway has been associated with many types of human disease, how the Hippo pathway becomes dysregulated is not well understood. In this study we created knockout cell lines for many different Hippo pathway components and compared their response to multiple stimuli to determine which components are the most physiologically important in regulating YAP/TAZ.

Comparing the core Hippo pathway components, it is clear that in response to all stimuli and conditions tested, in HEK293A cells, LATS1/2 are the primary direct kinases

for YAP/TAZ. Although LATS1/2 may have some intrinsic kinase activity towards YAP/TAZ, MOB1A/B are required for full phosphorylation and activation of LATS1/2. MST1/2 are the primary kinases for MOB1A/B, but in the MST1/2 KO cells, YAP/TAZ are still significantly phosphorylated following serum starvation even without phosphorylated MOB1A/B (T35) (Figure 3.5E), indicating that MOB1A/B phosphorylation is not essential for LATS1/2 activation. Moreover, MOB1A/B phosphorylation is not sufficient to induce LATS1/2 phosphorylation, as LATS1/2 (HM) phosphorylation is absent in the NF2 KO cells even though MOB1A/B (T35) phosphorylation is high (Figure 3.5E). In addition, YAP phosphorylation is severely compromised in the MOB1A/B KO cells despite relatively high LATS1/2 (HM) phosphorylation, suggesting that LATS1/2 (HM) phosphorylation by itself is not sufficient and MOB1A/B plays a critical role in LATS1/2 phosphorylation of YAP. Together, these findings demonstrate that while MOB1A/B is critical for full LATS1/2 phosphorylation and activation, phosphorylation of MOB1A/B (T35) is not essential. Furthermore, phosphorylation of LATS1/2 (HM) is not sufficient to predict LATS1/2 activity. The MAP4K family and TAOK1/3 can directly interact with and phosphorylate LATS1/2 (HM) (Li et al., 2014a; Meng et al., 2015; Zheng et al., 2015), but this is still dependent on MOB1A/B to phosphorylate YAP/TAZ. These data fit with the model that MOB1A/B promotes YAP/TAZ phosphorylation by LATS1/2 and is required for auto-phosphorylation of the LATS1/2 activation loop. Thus, LATS1/2 are not fully active even though the HM is phosphorylated when MOB1A/B is absent.

The small increase in phosphorylated YAP in the MOB1A/B KO cells in response to cell-cell contact may be due to enhanced intrinsic activity of LATS1/2 (for example, if

LATS1/2 and YAP/TAZ are both localized near adherens junctions at high density to further facilitate YAP/TAZ phosphorylation). Of note, with increasing density, MOB1A/B (T35) phosphorylation is significantly decreased while phosphorylated LATS1/2 (HM) and YAP (S127) are increased (Figure 3.4C). These observations again demonstrate that phosphorylated MOB1A/B (T35) is not required for LATS1/2 activation.

NF2 deletion is sufficient to significantly disrupt YAP/TAZ phosphorylation. Although we did not observe any change in LATS1/2 protein stability, it is clear that NF2 plays a critical role in LATS1/2 phosphorylation and activation. Deleting NF2 abolished YAP/TAZ phosphorylation in response to serum starvation. The current model for LATS1/2 activation begins when MST1/2 is recruited to the plasma membrane and phosphorylated. Phosphorylated MST1/2 binds and phosphorylates MOB1A/B, and NF2 recruits LATS1/2 to plasma membrane where LATS1/2 joins the MST1/2-MOB1A/B complex and are phosphorylated and activated by MST1/2 (Yin et al., 2013). The mechanism of how NF2 recruits LATS1/2 to the plasma membrane is not fully understood, but as our data demonstrate, NF2 plays a key role in LATS1/2 activation but not MST1/2 kinase activity because MOB1A/B (T35) was fully phosphorylated in the NF2 KO cells. NF2 probably plays an important role in MAP4K and TAOK-mediated activation of LATS1/2 as well because deleting NF2 had a more severe effect on YAP phosphorylation than deleting MST1/2. This role is also likely due to localization, since deletion of NF2 did not affect TAOK1 kinase activity (Figure 3.6D). There is not a reliable phospho-MAP4K antibody available to determine endogenous MAP4K phosphorylation. NF2 is one of a few instances where a mutation in a Hippo pathway component has a direct link to human disease. However, the observation that YAP/TAZ

can still be phosphorylated and that NF2 KO cells retain some sensitivity to certain types of stress gives hope that there may be ways to therapeutically inhibit YAP/TAZ in NF2-mutant patients.

We have identified TAOK1/3 as direct kinases for LATS1/2. Previous studies have shown that TAOK phosphorylates and activates MST1/2 (Boggiano et al., 2011; Poon et al., 2011). Together, we propose that TAOK acts not only upstream of but in parallel to MST1/2 and the MAP4Ks to stimulate LATS1/2. Deleting TAOK1/2/3 in the MST1/2-MAP4K4/6/7 5KO cells significantly reduced YAP/TAZ phosphorylation and cytoplasmic localization in response to serum starvation and cell-cell contact. Although MST1/2 and the MAP4K family account for the majority of LATS1/2 phosphorylation and activation in response to most stimuli tested, TAOK1/3 played a significant role in response to cell-cell contact. The MST-MAP4K-TAOK 8KO and LATS1/2 KO cells are the only cell lines tested resistant to YAP phosphorylation by cell-cell contact. This raises the possibility that TAOK may have a significant role in regulating LATS1/2 activity in response to other stress conditions as well. That there are at least three distinct kinase families (MST, MAP4K, TAOK) capable of activating LATS1/2 highlights: (1) how critical regulation of LATS1/2 and YAP/TAZ are, and (2) how complex regulation of the Hippo pathway is that the cell has evolved so many mechanisms to tightly regulate YAP/TAZ in response to many different stimuli and contexts.

Finally, we establish a clear role for RHOA in mediating growth signals from GPCRs to activate YAP/TAZ. When we deleted RHOA, YAP/TAZ remained highly phosphorylated, sequestered in the cytoplasm, and transcriptionally inactive even in the presence of serum. However, TPA stimulation induced TAZ dephosphorylation and

YAP/TAZ nuclear localization, raising the possibility that there are conditions in which YAP and TAZ may be differentially regulated (Figure 3.7D and 3.7E). To our knowledge, this is the first genetic data supporting a clear role for RHOA in mediating growth signals to the Hippo pathway, and illustrates how activating or inactivating mutations in components upstream of the Hippo pathway can result in pathological disruption of YAP/TAZ.

In conclusion, we compared and identified which Hippo pathway components have the greatest impact on regulating YAP/TAZ, and clarified their relationships with the core Hippo pathway kinase cascade (Figure S3.7). In addition, this study provides many useful resources for those studying the Hippo field, cell growth and survival, or the mechanisms of action of drugs targeting the Hippo pathway. It should be noted that our studies were only conducted in HEK293A cells, and it is possible that some components are not essential in HEK293A cells but are essential in other cell types. While we have clarified the role of NF2, TAOK1/3, and RHOA, there remain many other potential components whose deletion or overexpression may contribute to dysregulation of YAP/TAZ in human disease. Understanding these relationships will be critical to grasp how the Hippo pathway becomes disrupted and to identify potential therapeutic targets.

3.4 Experimental procedures

Generation of knockout cell lines

pSpCas9(BB)-2A-Puro (PX459; Addgene plasmid #48139) was a gift from Dr. Feng Zhang (Sanjana et al., 2014). Gene-specific sgRNAs were designed using the

CRISPR design tool at http://www.genome-engineering.org/crispr. HEK293A cells were transfected, selected with puromycin for 2-3 days, and single-cell sorted by FACs into 96-well plate format. Single clones were expanded and screened by protein immunoblot and confirmed by sequencing (Figure S3.1). All sgRNA sequences are listed in Table S3.1.

Cell culture

HEK293A cells were grown in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were plated at 1.5 x 10^5 cells per well into 6-well plates, unless otherwise noted. 24 hours after plating, cells were given fresh media for 2 hours before treatment with serum-free DMEM media, Latrunculin B (250 ng/ml), F/IBMX (10 uM Forskolin, 100 uM IBMX), or 2-DG (25 mM, in glucose-free DMEM with 10% dialyzed FBS). Cells were starved in serum-free DMEM overnight before treatment with LPA (0.5 uM) or TPA (5 nM). To measure glucose and pH, cells were plated at 8 x 10^5 cells per well into 6-well plates and given fresh DMEM with 10% FBS for 6 hours. Glucose was measured using a FreeStyle Precision Neo glucose monitoring system.

Immunoblot

Immunoblots were performed as previously described (Meng et al., 2015). 7.5% phos-tag gels were used to compare YAP and TAZ phosphorylation levels. Immunoprecipitation and in vitro kinase assays were performed as previous described (Meng et al., 2015). Antibodies used are listed in Table S3.2.

Immunofluorescence

Coverslips were pretreated with 0.0005% Poly-L-ornithine solution (Sigma, P4957) in 12-well plates at 37°C for 15 minutes and washed with PBS prior to plating cells. Cells were plated 24 hours prior to treatment: medium cell density (1.0×10^5), low cell density (0.5×10^5), serum starvation (12 hours), LPA (0.5 uM), or TPA (5 nM). Cells were fixed in 4% paraformaldehyde for 15 minutes, followed by permeabilization with 0.1% Triton-X for 5 minutes and blocking in 3% BSA for 1 hour. Primary antibody was incubated in 3% BSA overnight at 4°C. Secondary antibodies were diluted in 3% BSA and incubated for 1 hour. Slides were mounted with prolong gold anti-fade reagent with DAPI.

Statistical analysis

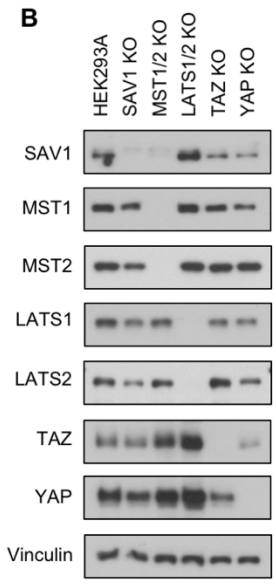
Where indicated, experiments were repeated at least three times and statistical analysis was performed using unpaired t tests. ns: $P>0.05$; *: $P\leq0.05$; **: $P\leq0.01$; ***: $P\leq0.001$; ****: $P\leq0.0001$.

3.5 Acknowledgments

Chapter 3, in full, is a reprint of the material as it appears in Molecular Cell; Plouffe, S.W., Meng, Z., Lin, K.C., Lin, B., Hong, A.W., Chun, J.V., Guan, K.L. Cell Press, 2016. The dissertation author was the primary investigator and author of this paper.

A

Cell Line	Genes
SAV1 KO	SAV1
MST1/2 KO	STK4, STK3
MAP4K4/6/7 KO	MAP4K4, MINK1, TNIK
MST1/2-MAP4K4/6/7 5KO	STK4, STK3, MAP4K4, MINK1, TNIK
MST-MAP4K 8KO	STK4, STK3, MAP4K1, MAP4K2, MAP4K3, MAP4K4, MINK1, TNIK
MOB1A/B KO	MOB1A, MOB1B
LATS1/2 KO	LATS1, LATS2
YAP KO	YAP
TAZ KO	WWTR1
YAP/TAZ KO	YAP, WWTR1
TAOK1/2/3 KO	TAOK1, TAOK2, TAOK3
MST-MAP4K-TAOK 8KO	STK4, STK3, MAP4K4, MINK1, TNIK, TAOK1, TAOK2, TAOK3
RHOA KO	RHOA
AMOT KO	AMOT
AJUBA KO	AJUBA
CTNNA1 KO	CTNNA1
NF2 KO	NF2
AMPK KO	PRKKA1, PRKAA2
RASSF1A KO	RASSF1A
PKA KO	PRKACA, PRKCB



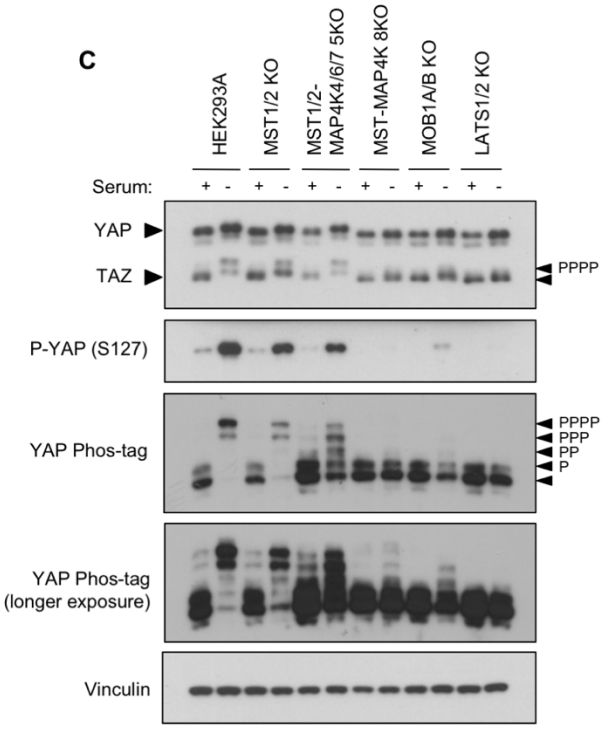


Figure 3.1. Using CRISPR to Target the Hippo Pathway.
(A) List of cell lines created and genes deleted in this study. See Table S2.1 and Figure S3.1 for sequences and immunoblots. (B) Immunoblots showing CRISPR-mediated deletion of core Hippo pathway components. (C) Overnight serum starvation induces YAP/TAZ phosphorylation and degradation in wild-type HEK293A cells. See Figure 3.2 for quantification. See Figure S3.7 for a schematic of the Hippo pathway.

Figure 3.2. YAP/TAZ Phosphorylation in Response to Serum Starvation.
(A–D) Immunoblots showing YAP/TAZ phosphorylation status following serum starvation in cells with deletions of either Hippo pathway core components (A), TAOK1/2/3 (B), Hippo pathway interactors (C), or RHOA and NF2 (D). The HEK293A cells in (A) are the relevant control for all panels; the figure is subdivided for readability. See Figure S3.3 for quantification.

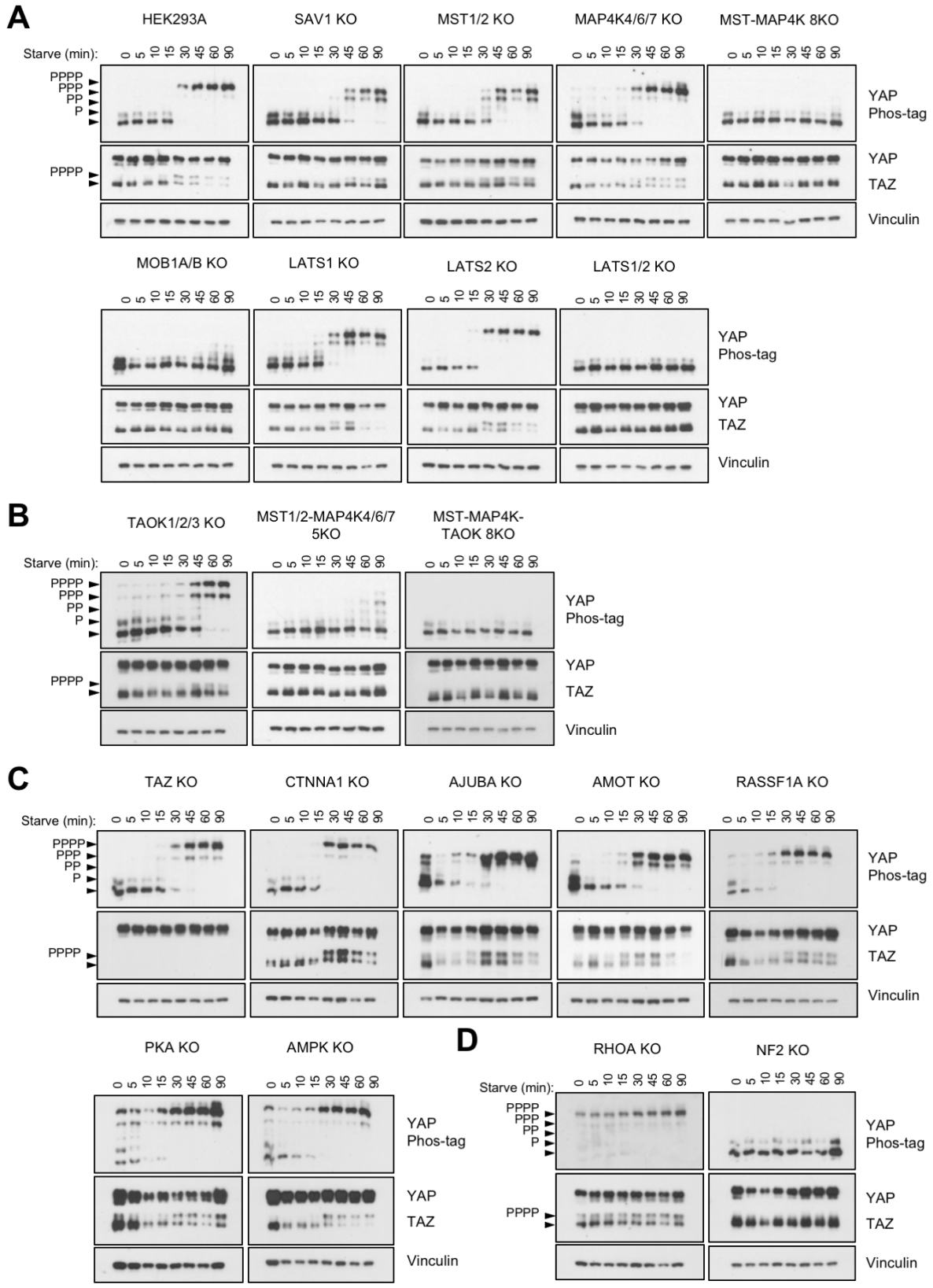

A
HEK293A
SAV1 KO
MST1/2 KO
MAP4K4/6/7 KO
MST-MAP4K 8KO
Starve (min): 0 5 10 15 30 45 60 90
PPPP
PPP
PP
P
YAP Phos-tag
YAP
TAZ
Vinculin
MOB1A/B KO
LATS1 KO
LATS2 KO
LATS1/2 KO
B
TAOK1/2/3 KO
MST1/2-MAP4K4/6/7 5KO
MST-MAP4K-TAOK 8KO
C
TAZ KO
CTNNA1 KO
AJUBA KO
AMOT KO
RASSF1A KO
PKA KO
AMPK KO
D
RHOA KO
NF2 KO

Figure 3.3. Dysregulation of YAP/TAZ Phosphorylation Results in Aberrant YAP/TAZ Localization and Transcriptional Activity.
(A) Immunofluorescence staining for YAP/TAZ (red) and DAPI (blue) in the presence of serum or following 12 hr of serum starvation. See Figure S3.4A for quantification. (B and C) Relative expression of YAP/TAZ downstream target genes CYR61 and CTGF in the presence of serum (B), or following overnight serum starvation (C), as quantified by qPCR. Data are represented as mean ± SD. (D and E) Glucose levels (D) and pH (E) of the culture media following a 6 hr incubation under normal culture conditions. Data are represented as mean ± SD.

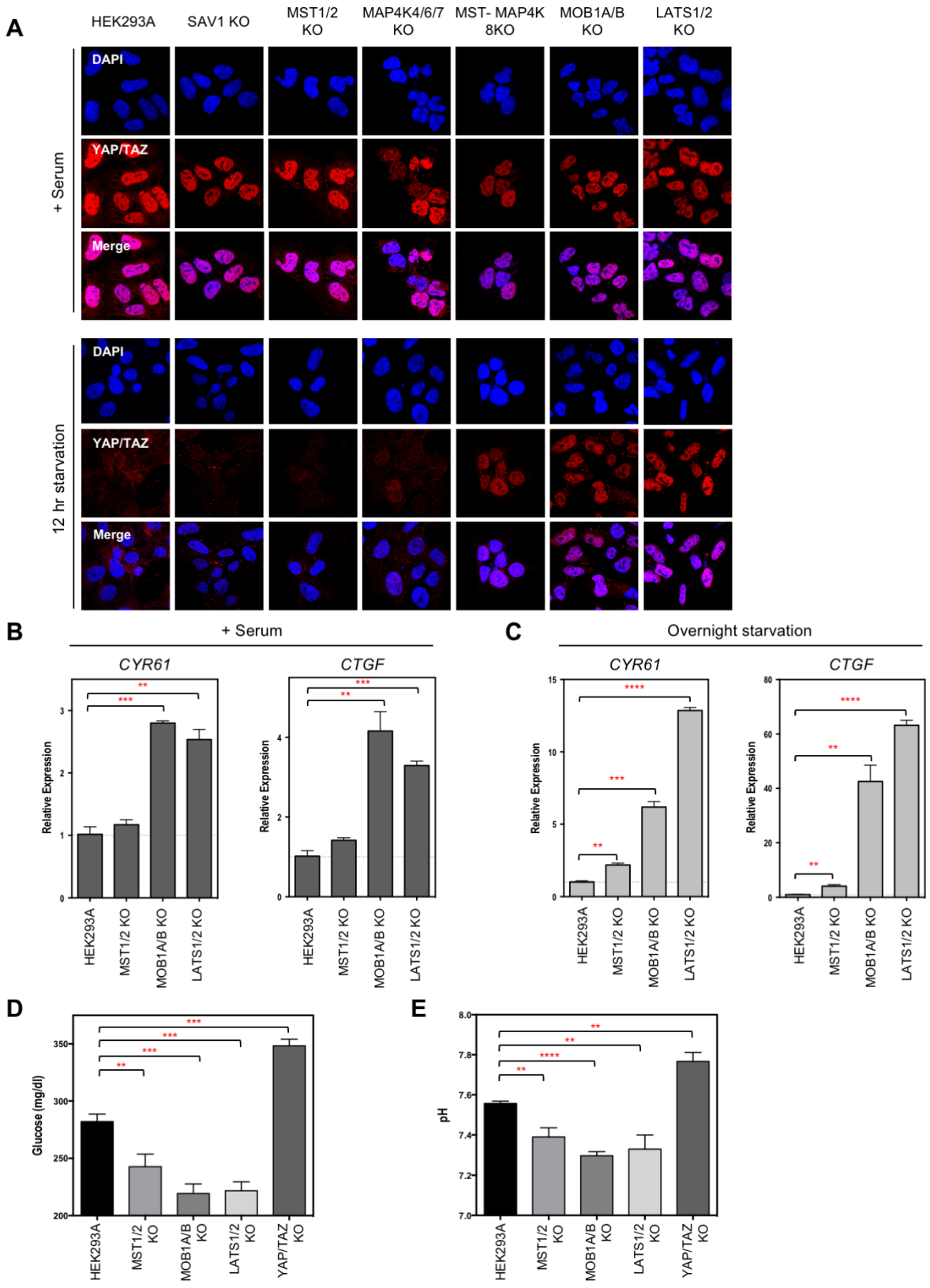

A
HEK293A
SAV1 KO
MST1/2 KO
MAP4K4/6/7 KO
MST- MAP4K 8KO
MOB1A/B KO
LATS1/2 KO
+ Serum
12 hr starvation
DAPI
YAP/TAZ
Merge
B
+ Serum
CYR61
CTGF
Relative Expression
C
Overnight starvation
D
Glucose (mg/dl)
E
pH
HEK293A
MST1/2 KO
MOB1A/B KO
LATS1/2 KO
YAP/TAZ KO

Figure 3.4. Inactivation of YAP/TAZ in Response to Cell-Cell Contact.
(A) Cells were plated at low, medium, and high densities in a 6-well plate. Images show wild-type cells at each of the respective densities. (B) Cell-cell contact induces YAP phosphorylation. Cells were plated at each of the respective densities and harvested 24 hr later. (C) YAP/TAZ phosphorylation in the wild-type, MST-MAP4K 8KO, MOB1A/B KO, and LATS1/2 KO cells in response to cell-cell contact. (D) Immunofluorescence staining for YAP/TAZ (red) and DAPI (blue) at low and medium densities. See Figure S3.4B for quantification. (E) Images show cellular morphologies of wild-type and CTNNA1 KO cells. Cells were plated at low density, and images were taken 24 hr later.

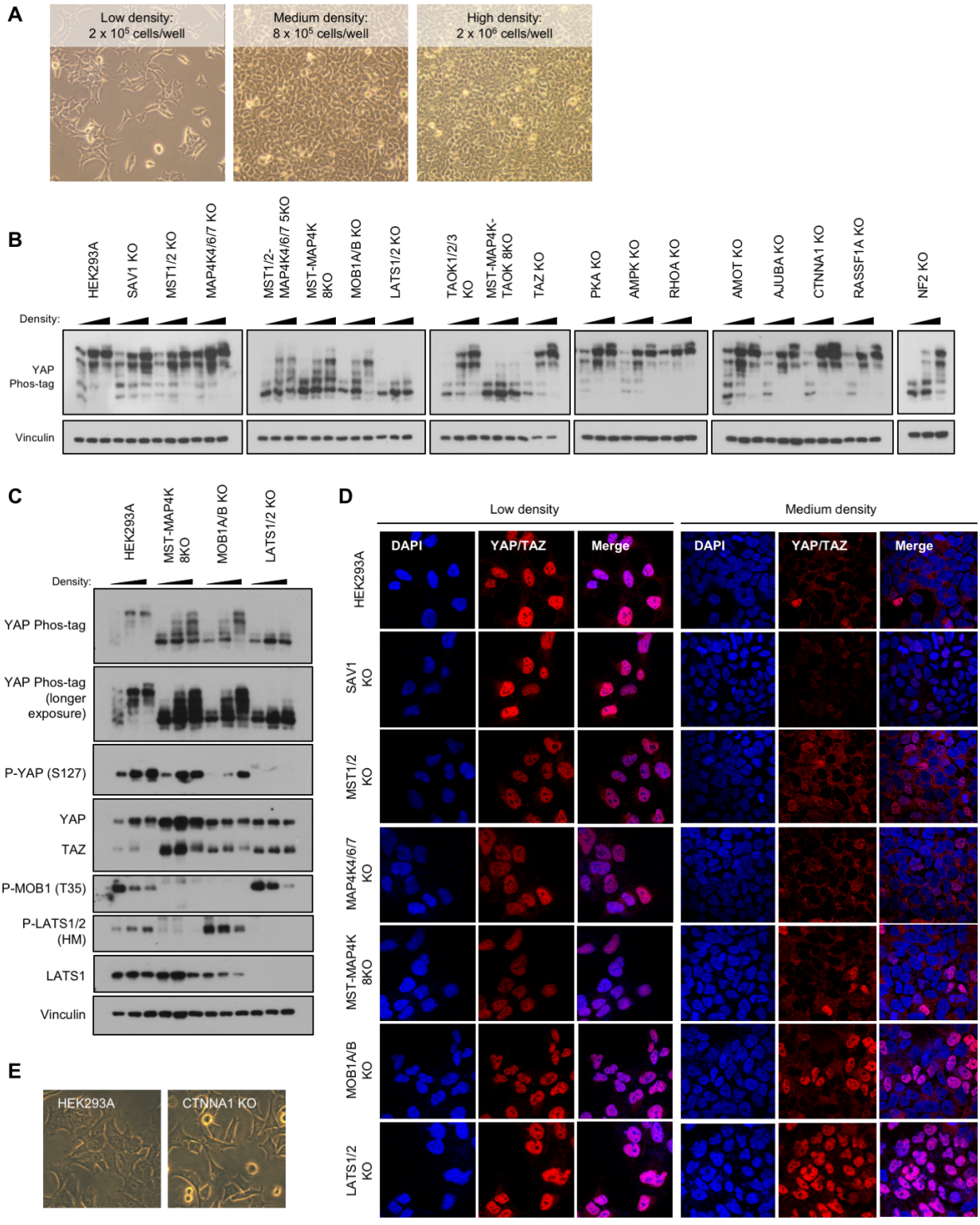

A
Low density:
2 x 10^5 cells/well
Medium density:
8 x 10^5 cells/well
High density:
2 x 10^6 cells/well
B
HEK293A
SAV1 KO
MST1/2 KO
MAP4K4/6/7 KO
MST1/2-MAP4K4/6/7 5KO
MST-MAP4K 8KO
MOB1A/B KO
LATS1/2 KO
TAOK1/2/3 KO
MST-MAP4K-TAOK 8KO
TAZ KO
PKA KO
AMPK KO
RHOA KO
AMOT KO
AJUBA KO
CTNNA1 KO
RASSF1A KO
NF2 KO
Density:
YAP Phos-tag
Vinculin
C
HEK293A
MST-MAP4K 8KO
MOB1A/B KO
LATS1/2 KO
Density:
YAP Phos-tag
YAP Phos-tag (longer exposure)
P-YAP (S127)
YAP
TAZ
P-MOB1 (T35)
P-LATS1/2 (HM)
LATS1
Vinculin
D
Low density
Medium density
DAPI
YAP/TAZ
Merge
HEK293A
SAV1 KO
MST1/2 KO
MAP4K4/6/7 KO
MST-MAP4K 8KO
MOB1A/B KO
LATS1/2 KO
E
HEK293A
CTNNA1 KO

Figure 3.5. Deletion of NF2 Results in Hyper-activated YAP/TAZ.
(A) Immunofluorescence staining for YAP/TAZ (red) and DAPI (blue) in wild-type and NF2 KO cells at low (LD) and medium (MD) densities in the presence of serum. See Figure S3.4C for quantification. (B and C) Relative expression of CYR61 and CTGF in the presence of serum (B) or following overnight serum starvation (C), as quantified by qPCR. Data are represented as mean ± SD. (D) Immunofluorescence staining for YAP/TAZ (red) and DAPI (blue) in wild-type and NF2 KO cells at low and medium densities following 12 hr serum starvation. See Figure S3.4C for quantification. (E) Deletion of NF2 prevents LATS1/2 (HM) phosphorylation in response to overnight serum starvation. (F) Protein stability of LATS1/2 is not affected in NF2 KO cells following treatment with 100 ug/ml cycloheximide. (G) Deletion of NF2 confers a growth advantage in HEK293A cells. Cells were plated at 7 × 104 cells/well in a 6-well plate with fresh media and counted after 0, 24, 48, 72, and 96 hr. Data are represented as mean ± SD. (H) NF2 KO cells are sensitive to actin disruption by Latrunculin B. See Figure S3.5 for YAP/TAZ phosphorylation response of other cell lines. (I) NF2 KO cells are sensitive to cellular energy stress.

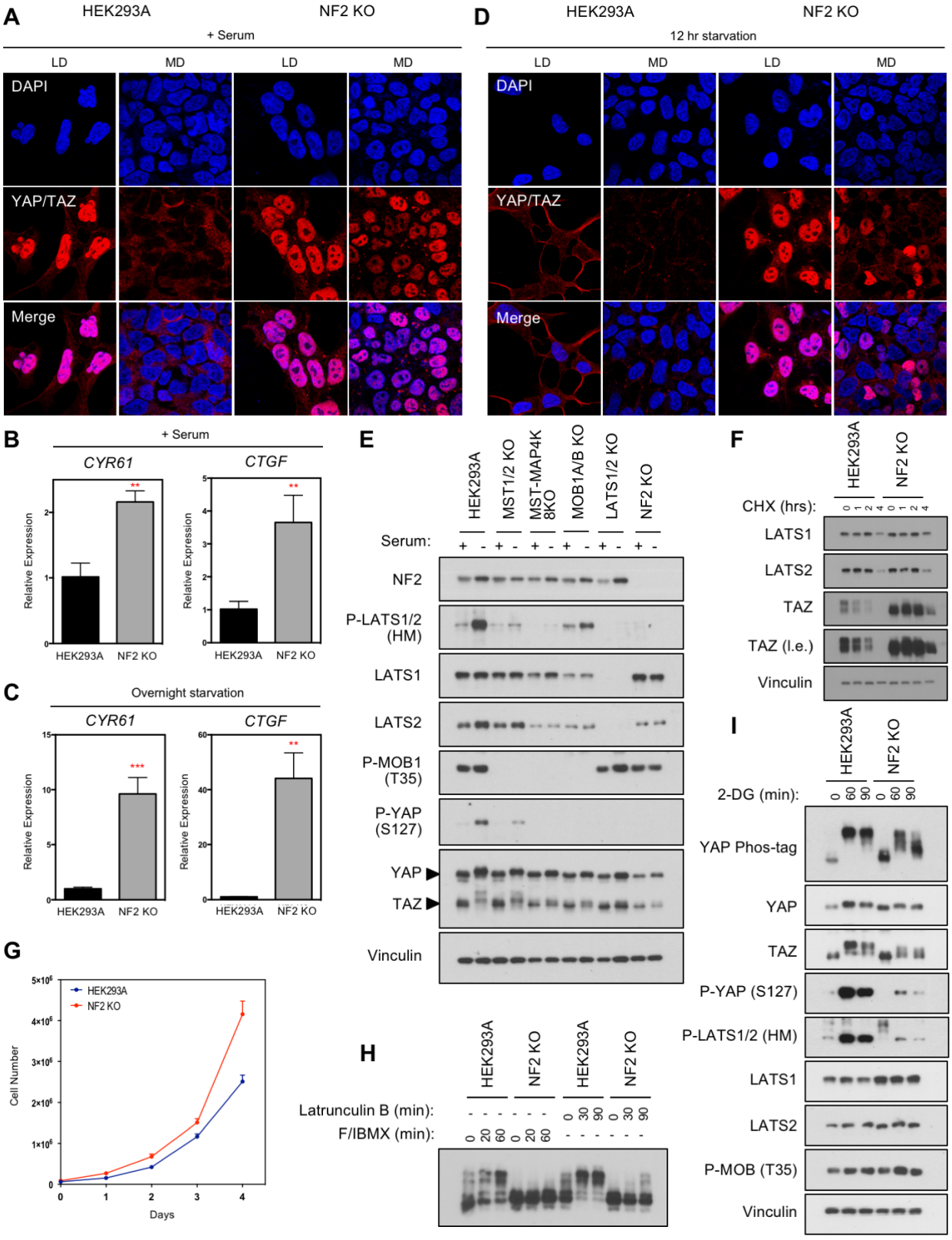

A
HEK293A
NF2 KO
+ Serum
LD
MD
LD
MD
DAPI
YAP/TAZ
Merge
D
HEK293A
NF2 KO
12 hr starvation
LD
MD
LD
MD
DAPI
YAP/TAZ
Merge
B
+ Serum
CYR61
CTGF
Relative Expression
HEK293A
NF2 KO
C
Overnight starvation
CYR61
CTGF
Relative Expression
HEK293A
NF2 KO
E
HEK293A
MST1/2 KO
MST-MAP4K 8KO
MOB1A/B KO
LATS1/2 KO
NF2 KO
Serum:
NF2
P-LATS1/2 (HM)
LATS1
LATS2
P-MOB1 (T35)
P-YAP (S127)
YAP
TAZ
Vinculin
F
HEK293A
NF2 KO
CHX (hrs):
LATS1
LATS2
TAZ
TAZ (l.e.)
Vinculin
G
HEK293A
NF2 KO
Cell Number
Days
H
HEK293A
NF2 KO
HEK293A
NF2 KO
Latrunculin B (min):
F/IBMX (min):
I
HEK293A
NF2 KO
2-DG (min):
YAP Phos-tag
YAP
TAZ
P-YAP (S127)
P-LATS1/2 (HM)
LATS1
LATS2
P-MOB (T35)
Vinculin

Figure 3.6. TAOK1/3 Are Direct Kinases for LATS1/2.
(A) Overexpression of TAOK1/3 induces LATS1/2 (HM) phosphorylation in wild-type cells. Cells were transfected with HA-LATS1 and various kinases including MST2, TAOK1, and TAOK3, and HA-LATS1 was immunoprecipitated and immunoblotted for HM phosphorylation. (B) TAOK1/3 can directly phosphorylate LATS1/2 (HM) in an in vitro kinase assay. TAOK1, TAOK2, and TAOK3 were transfected into wild-type cells and immunoprecipitated, and an in vitro kinase assay was performed with a truncated form of LATS1 (aa 638–1,130). (C) Overexpression of TAOK1 induces YAP phosphorylation in the MST-MAP4K 8KO but not the LATS1/2 KO cells. Cells were seeded at 0.7 × 105 cells/well in a 6-well plate, transfected with TAOK1-MYC, and harvested 24 hr later. (D) TAOK kinase activity is unaffected in the NF2 KO cells. P38 is a downstream target of TAOK. (E) The MST1/2-MAP4K-TAOK 8KO cells are resistant to cell-cell contact. Immunofluorescence staining for YAP/TAZ (red) and DAPI (blue) at low and medium densities. See Figure S3.4D for quantification.

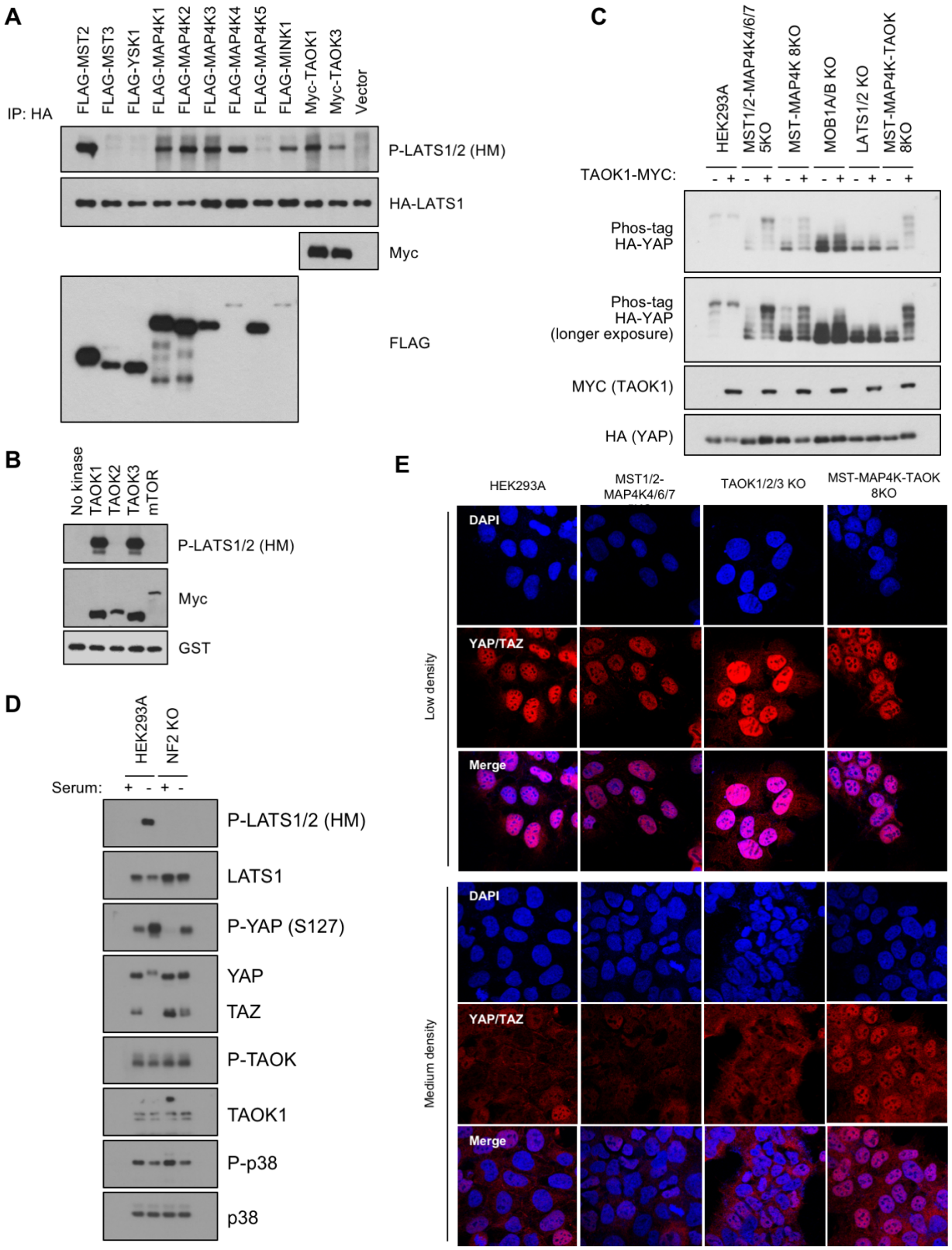

A
IP: HA
FLAG-MST2
FLAG-MST3
FLAG-YSK1
FLAG-MAP4K1
FLAG-MAP4K2
FLAG-MAP4K3
FLAG-MAP4K4
FLAG-MAP4K5
FLAG-MINK1
Myc-TAOK1
Myc-TAOK3
Vector
P-LATS1/2 (HM)
HA-LATS1
Myc
FLAG
B
No kinase
TAOK1
TAOK2
TAOK3
mTOR
P-LATS1/2 (HM)
Myc
GST
C
HEK293A
MST1/2-MAP4K4/6/7 5KO
MST-MAP4K 8KO
MOB1A/B KO
LATS1/2 KO
MST-MAP4K-TAOK 8KO
TAOK1-MYC:
- + - + - + - + - + - +
Phos-tag HA-YAP
Phos-tag HA-YAP (longer exposure)
MYC (TAOK1)
HA (YAP)
D
HEK293A
NF2 KO
Serum: + - + -
P-LATS1/2 (HM)
LATS1
P-YAP (S127)
YAP
TAZ
P-TAOK
TAOK1
P-p38
p38
E
HEK293A
MST1/2-MAP4K4/6/7
TAOK1/2/3 KO
MST-MAP4K-TAOK 8KO
Low density
Medium density
DAPI
YAP/TAZ
Merge

Figure 3.7. RHOA Is an Important Mediator of Growth Signals to Activate YAP/TAZ. (A) Treatment with the Rho inhibitor C3 exoenzyme (C3) induces YAP/TAZ phosphorylation. Cells were treated with C3 (1 ug/ml) for 4 hr. (B) The RHOA KO cells showed altered morphology compared to the wild-type cells. (C) Relative expression of CYR61 and CTGF in the presence of serum, as quantified by qPCR. Data are represented as mean ± SD. (D) LPA fails to induce YAP/TAZ dephosphorylation while TPA can induce TAZ dephosphorylation in the RHOA KO cells. Cells were starved overnight before treatment with LPA (0.5 uM) or TPA (5 nM). See Figure S3.6 for YAP/TAZ phosphorylation response of other cell lines. (E) Immunofluorescence staining for YAP/TAZ (red) and DAPI (blue) in the HEK293A wild-type and RHOA KO cells. See Figure S3.4E for quantification.

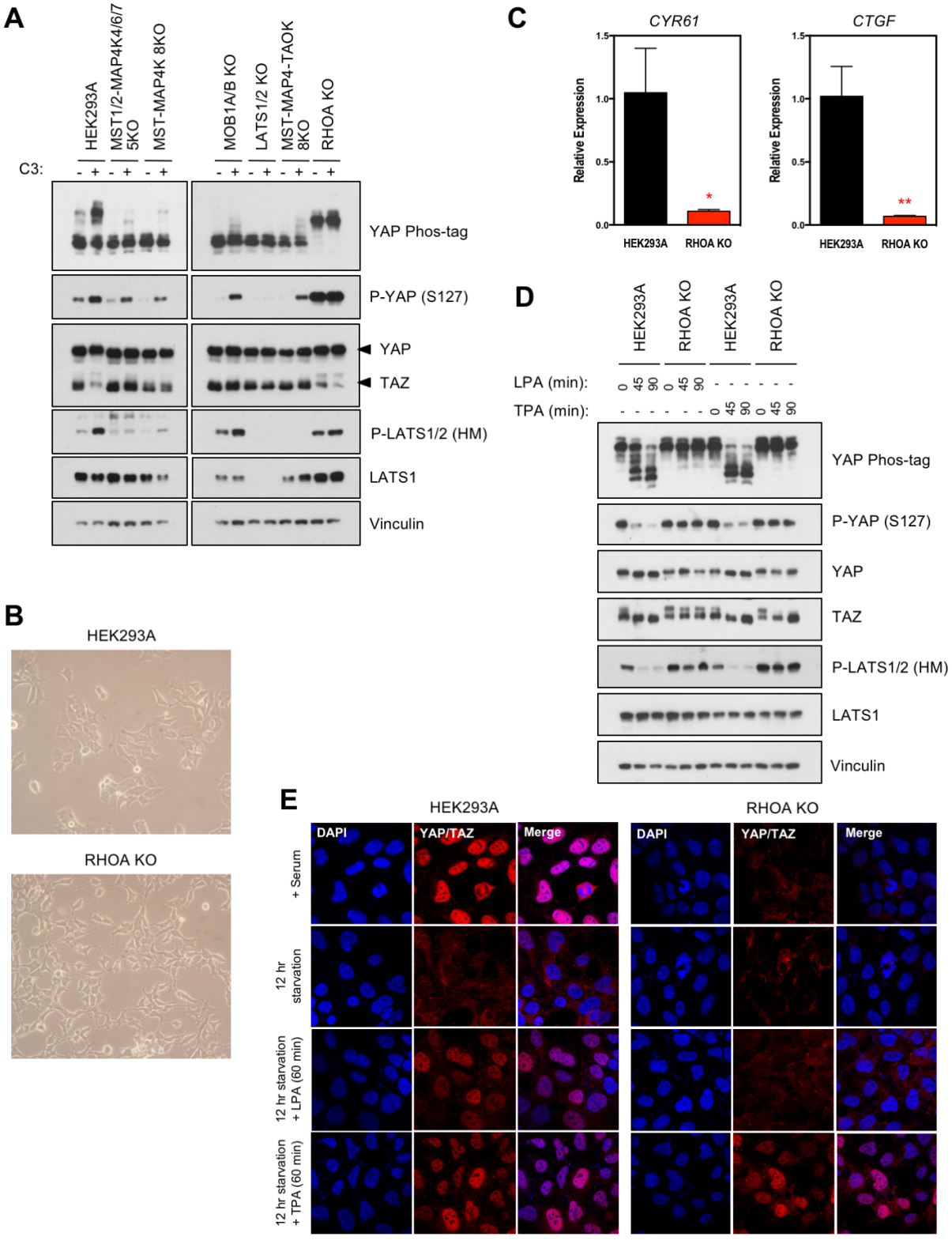
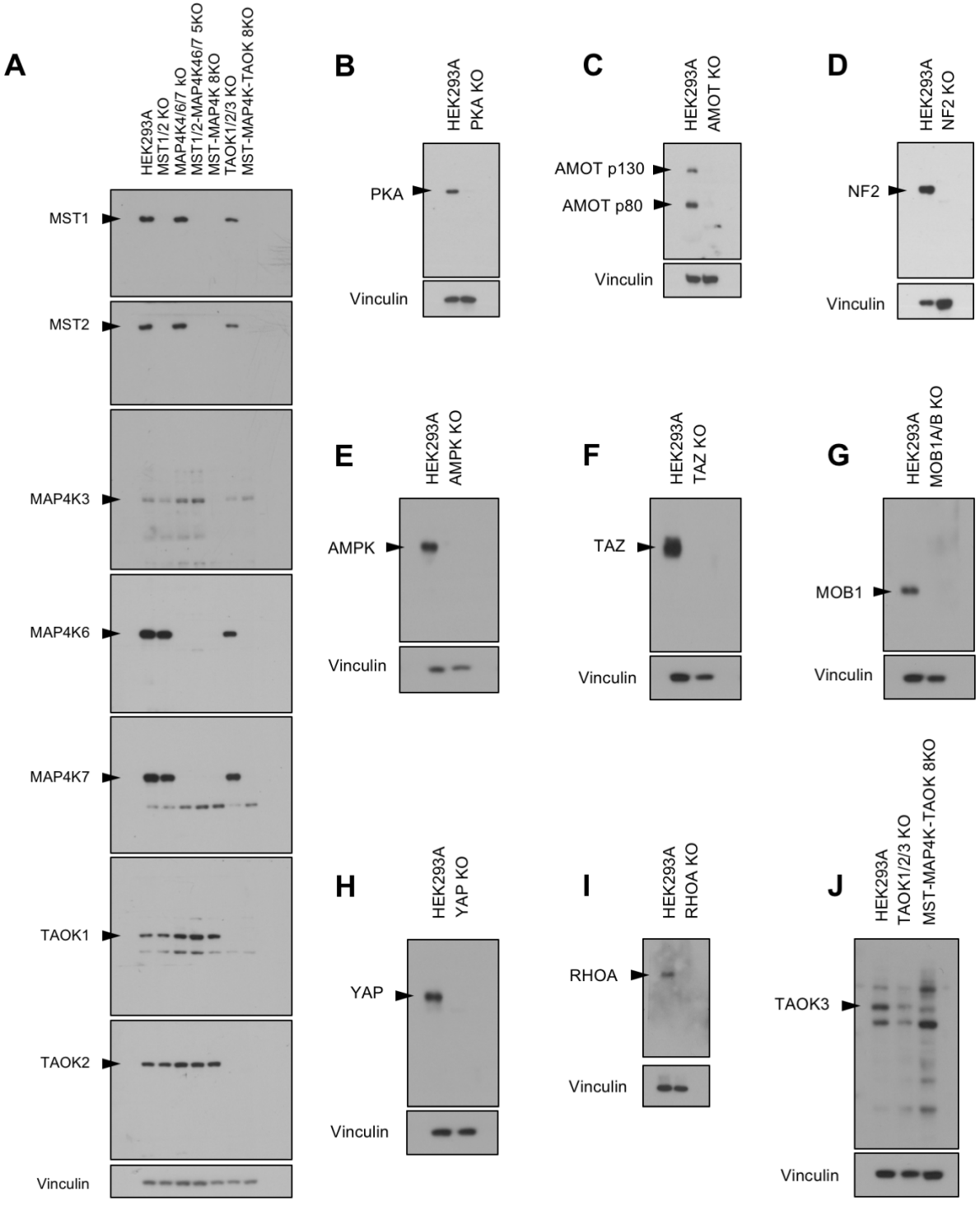

A
HEK293A
MST1/2-MAP4K4/6/7 5KO
MST-MAP4K 8KO
MOB1A/B KO
LATS1/2 KO
MST-MAP4-TAOK 8KO
RHOA KO
C3:
YAP Phos-tag
P-YAP (S127)
YAP
TAZ
P-LATS1/2 (HM)
LATS1
Vinculin
B
HEK293A
RHOA KO
C
CYR61
CTGF
Relative Expression
HEK293A
RHOA KO
D
LPA (min):
TPA (min):
E
HEK293A
RHOA KO
DAPI
YAP/TAZ
Merge
+ Serum
12 hr starvation
12 hr starvation + LPA (60 min)
12 hr starvation + TPA (60 min)

Figure S3.1. Immunoblots and sequences of targeted genes in this study.
A-J. Immunoblots showing knockout of MST1, MST2, MAP4K3, MAP4K6, MAP4K7, TAOK1, TAOK2, PKA, AMOT, NF2, AMPK, TAZ, MOB1A/B, YAP, and RHOA. TAOK3 antibody shows reduction of TAOK3 protein levels. K-M. Diagrams showing sgRNA target sites (in red) for proteins which have multiple isoforms but do not have usable antibodies. Although not all CTNNA1 isoforms are targeted, the dramatic change in morphology (Figure 3.4E) suggests a functional loss of CTNNA1. N-Y. Sequencing of AJUBA, CTNNA1, AMPK, NF2, RHOA, YAP, PKA, RASSF1A, TAZ, TAOK1, TAOK2, and MOB1. Some genes were not able to be conclusively sequenced due to multiple alleles or complex genomic DNA. Although YAP and PRKCB have alleles with in-frame deletions, immunoblots (B and H) show loss of protein expression. Z. RNA-seq of RHOA, RHOB, and RHOC in HEK293A cells (Sultan et al., 2008). AA-AL. Diagrams showing antibody recognition sites for proteins with multiple isoforms.
AN-AO. Re-expression experiments showing rescue of YAP regulation in the RHOA and MOB1A/B KO cells.


A
HEK293A
MST1/2 KO
MAP4K4/6/7 kO
MST1/2-MAP4K46/7 5KO
MST-MAP4K 8KO
TAOK1/2/3 KO
MST-MAP4K-TAOK 8KO
MST1
MST2
MAP4K3
MAP4K6
MAP4K7
TAOK1
TAOK2
Vinculin
B
HEK293A
PKA KO
PKA
Vinculin
C
HEK293A
AMOT KO
AMOT p130
AMOT p80
Vinculin
D
HEK293A
NF2 KO
NF2
Vinculin
E
HEK293A
AMPK KO
AMPK
Vinculin
F
HEK293A
TAZ KO
TAZ
Vinculin
G
HEK293A
MOB1A/B KO
MOB1
Vinculin
H
HEK293A
YAP KO
YAP
Vinculin
I
HEK293A
RHOA KO
RHOA
Vinculin
J
HEK293A
TAOK1/2/3 KO
MST-MAP4K-TAOK 8KO
TAOK3
Vinculin

K

L

Figure S3.1. Immunoblots and sequences of targeted genes in this study. (continued)

Figure S3.1. Immunoblots and sequences of targeted genes in this study. (continued)

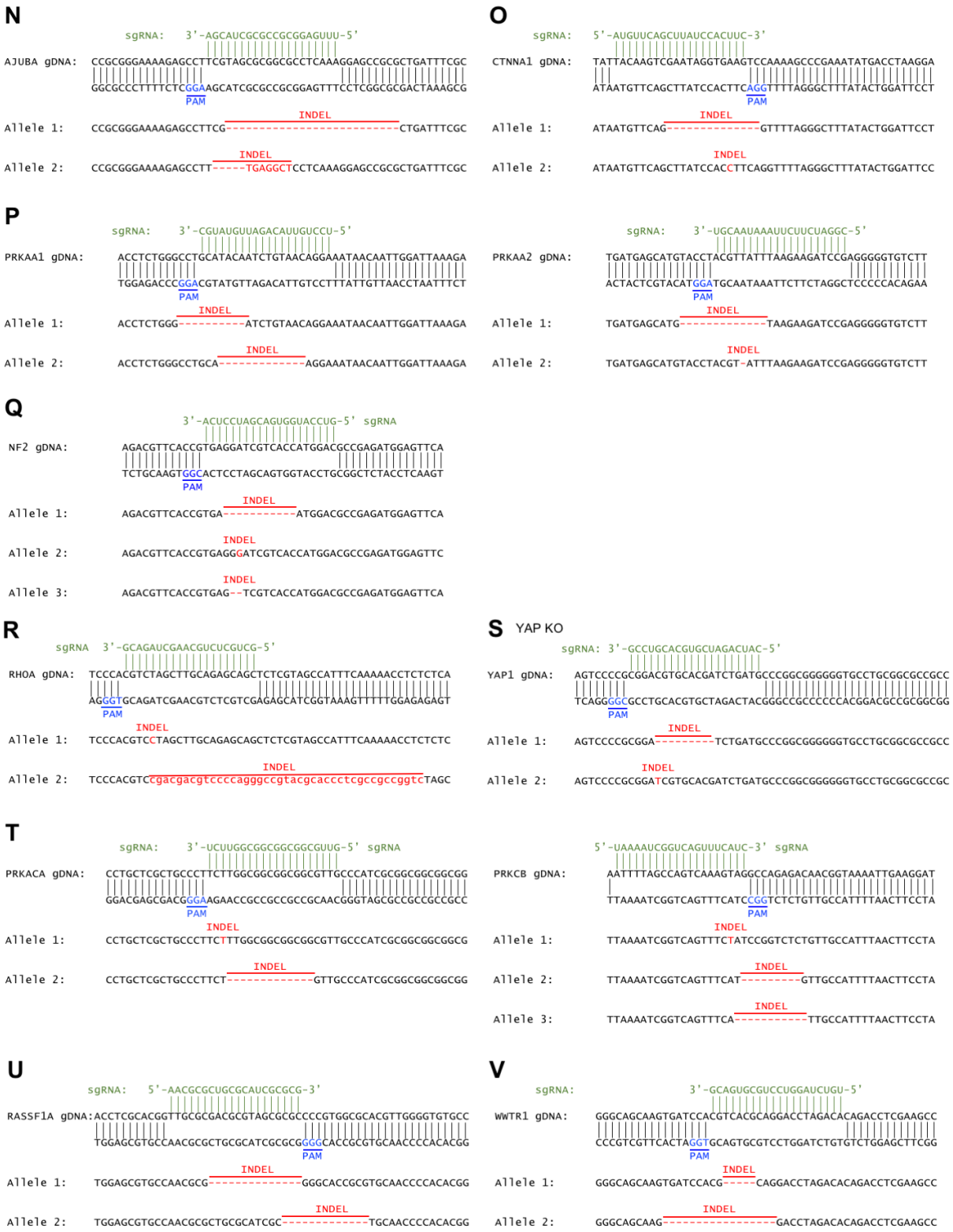


Figure S3.1. Immunoblots and sequences of targeted genes in this study. (continued)

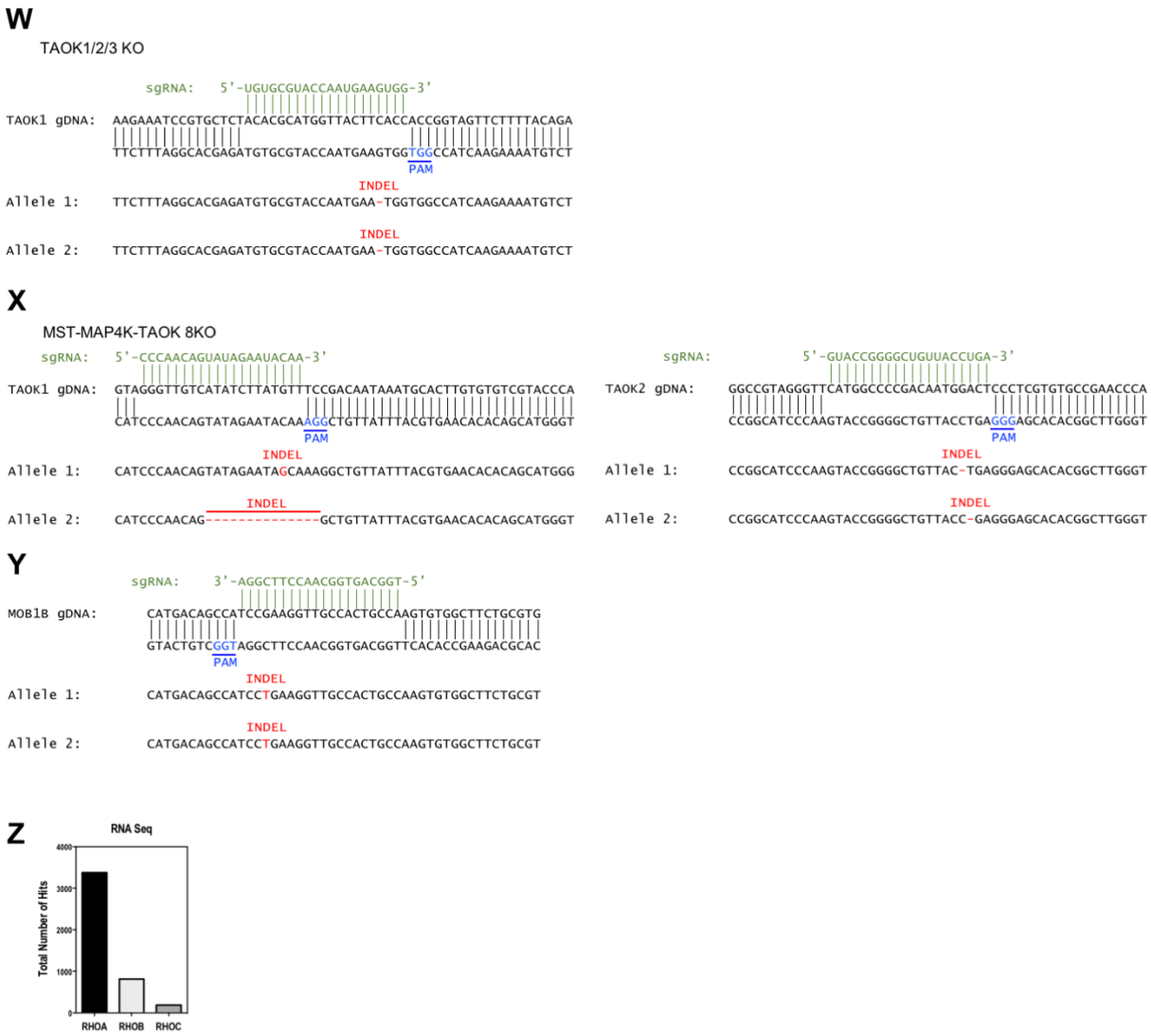


Figure S3.1. Immunoblots and sequences of targeted genes in this study. (continued)

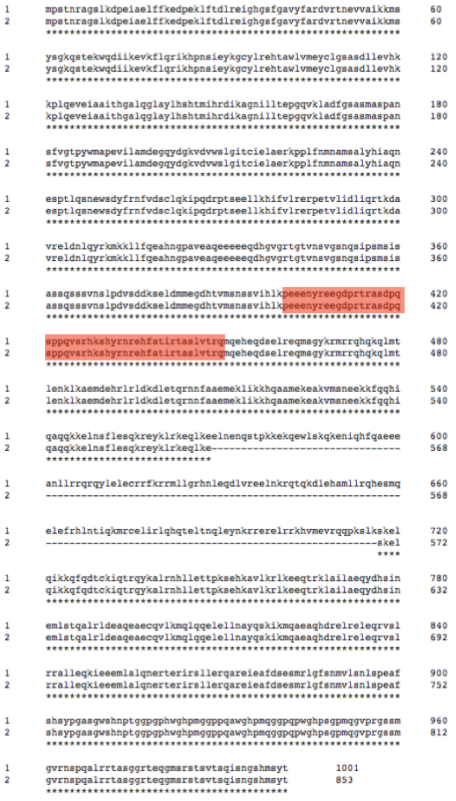
Figure S3.1. Immunoblots and sequences of targeted genes in this study. (continued)

Figure S3.1. Immunoblots and sequences of targeted genes in this study. (continued)

AG

TAOK1

1 – TAOK1 isoform 1 (NP_065842)
2 – TAOK1 isoform 2 (NP_079418)



AH

TAOK2

1 – TAOK2 isoform 1 (NP_057235)
2 – TAOK2 isoform 2 (NP_004774)
3 – TAOK2 isoform 3 (NP_001238972)

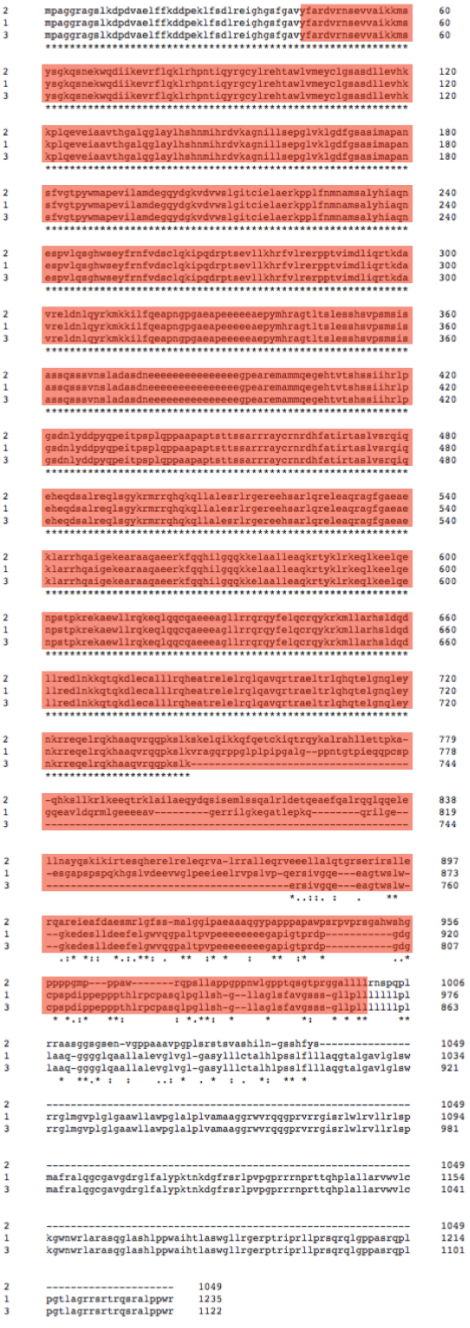


Figure S3.1. Immunoblots and sequences of targeted genes in this study. (continued)

AI

NF2

1 – NF2 isoform 1 (NP_000259)
2 – NF2 isoform 2 (NP_057502)
5 – NF2 isoform 5 (NP_861966)
6 – NF2 isoform 6 (NP_861967)
7 – NF2 isoform 7 (NP_861969)

AJ

YAP

1 – YAP isoform 1 (NP_001123617)
2 – YAP isoform 2 (NP_006097)
3 – YAP isoform 3 (NP_001181973)
4 – YAP isoform 4 (NP_001181974)
5 – YAP isoform 5 (NP_001269027)
6 – YAP isoform 6 (NP_001269026)
7 – YAP isoform 7 (NP_001269028)
8 – YAP isoform 8 (NP_001269029)
9 – YAP isoform 9 (NP_001269030)

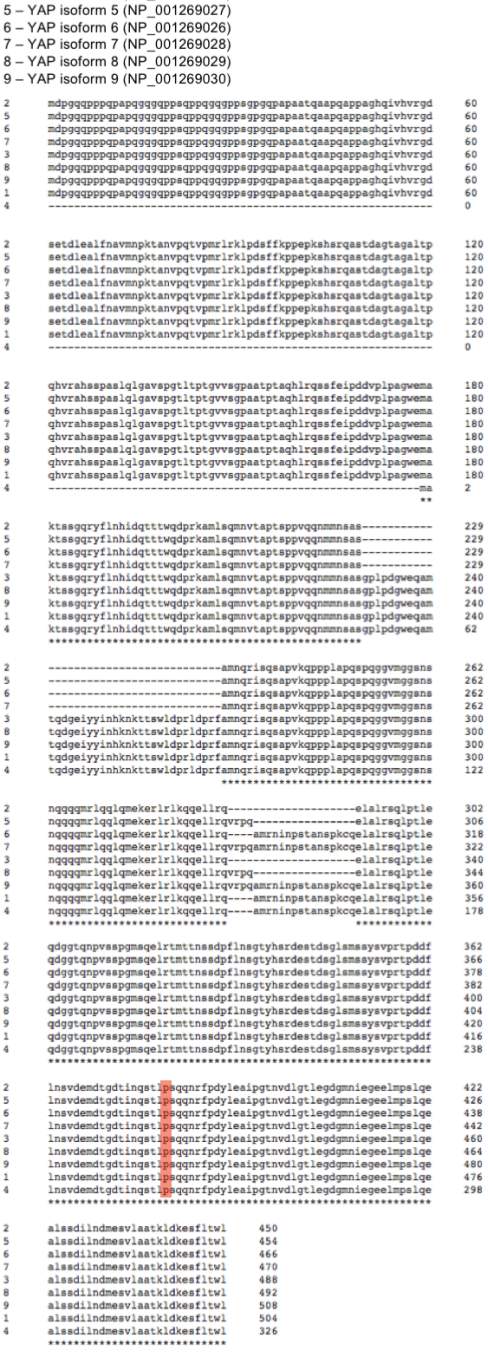


Figure S3.1. Immunoblots and sequences of targeted genes in this study. (continued)

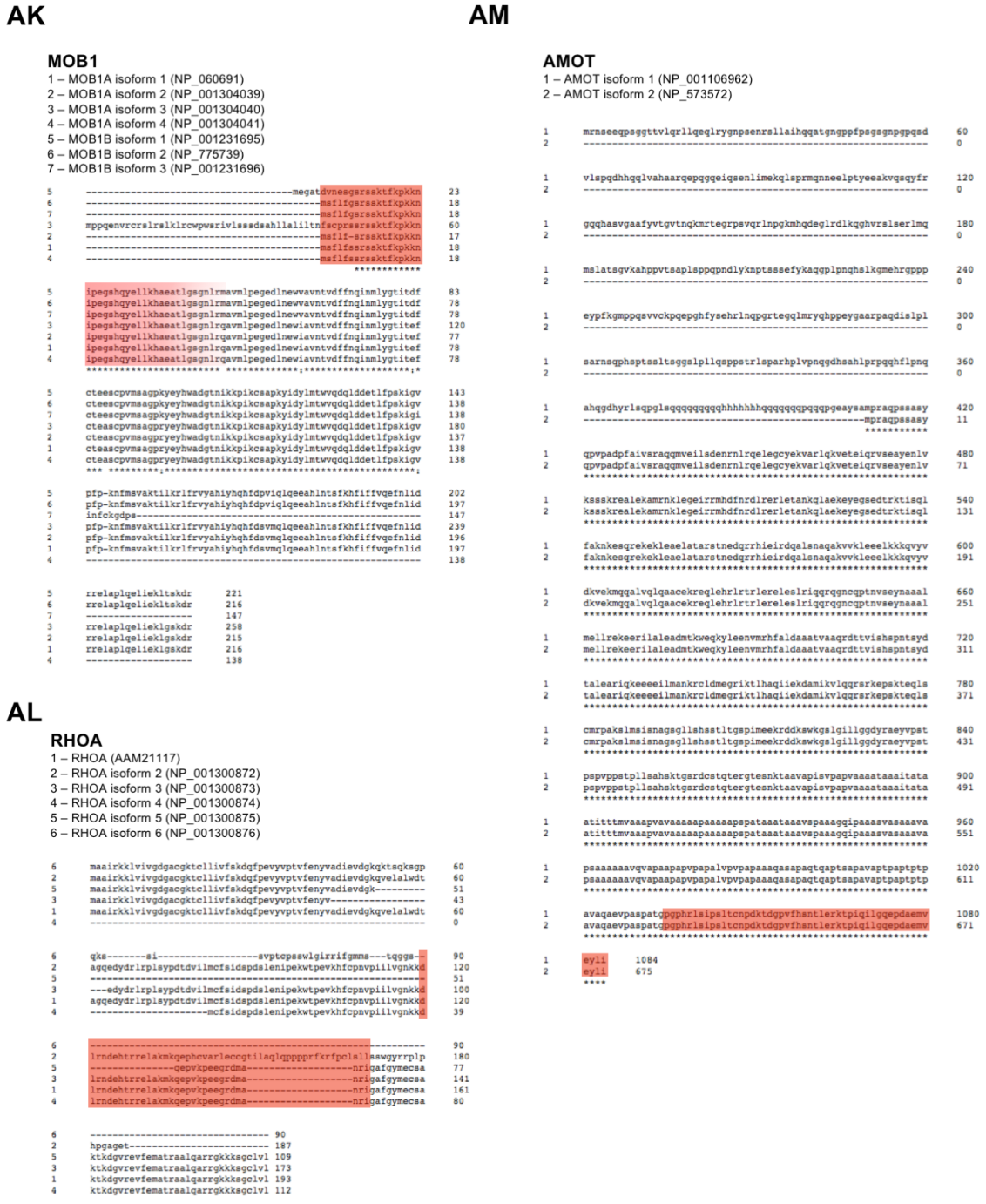


Figure S3.1. Immunoblots and sequences of targeted genes in this study. (continued)

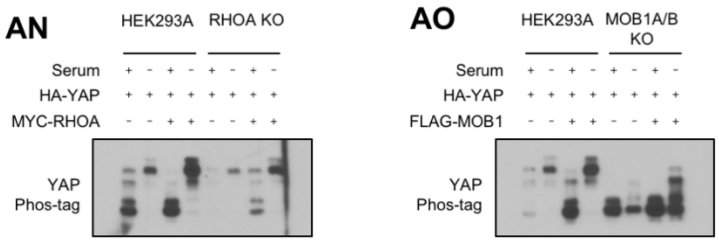
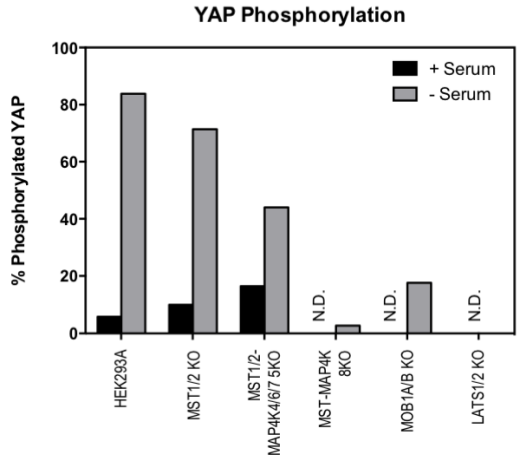


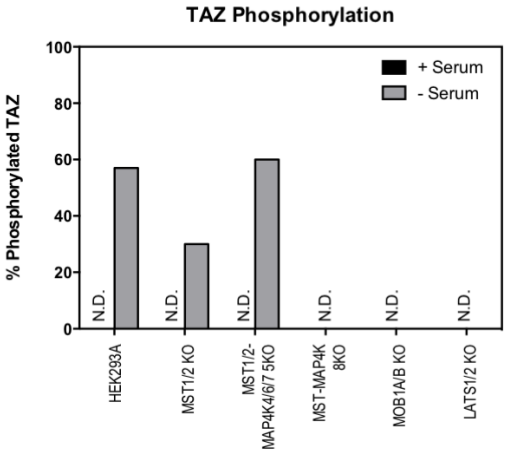
Figure S3.1. Immunoblots and sequences of targeted genes in this study. (continued)

Figure S3.2. Quantification of YAP/TAZ phosphorylation.
A. Quantification of phosphorylated YAP in Figure 3.1C. Data represented as mean +/- S.D. N.D. indicates not detected. B. Quantification of phosphorylated TAZ in Figure 3.1C. Data represented as mean +/- S.D. C. Phos-tag analysis of phosphorylated TAZ in the presence of serum or following overnight serum starvation. D. Quantification of phosphorylated YAP in Figure S3.2C. Data represented as mean +/- S.D. E. Quantification of phosphorylated TAZ in Figure S3.2C. Data represented as mean +/- S.D.

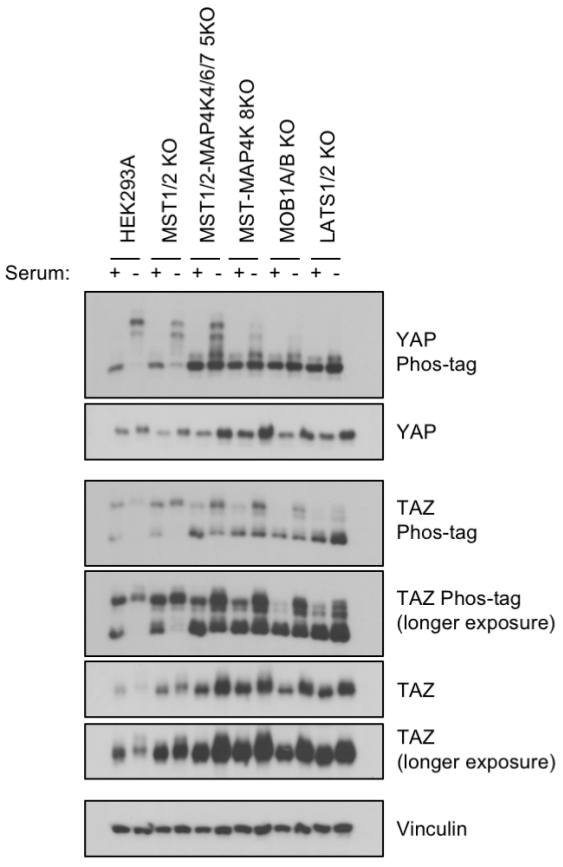
A Quantification from Figure 3.1C



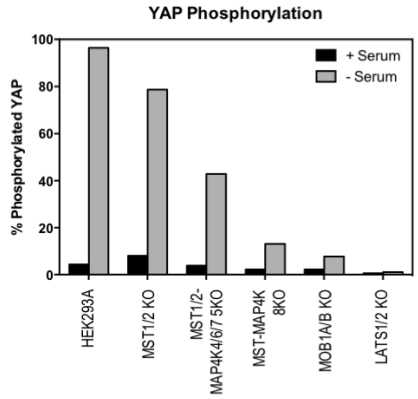
B Quantification from Figure 3.1C



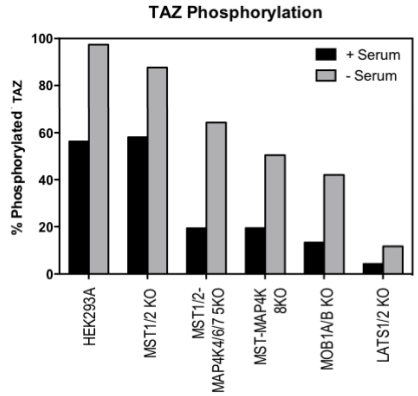
C



D Quantification from Figure S3.2C



E Quantification from Figure S3.2C



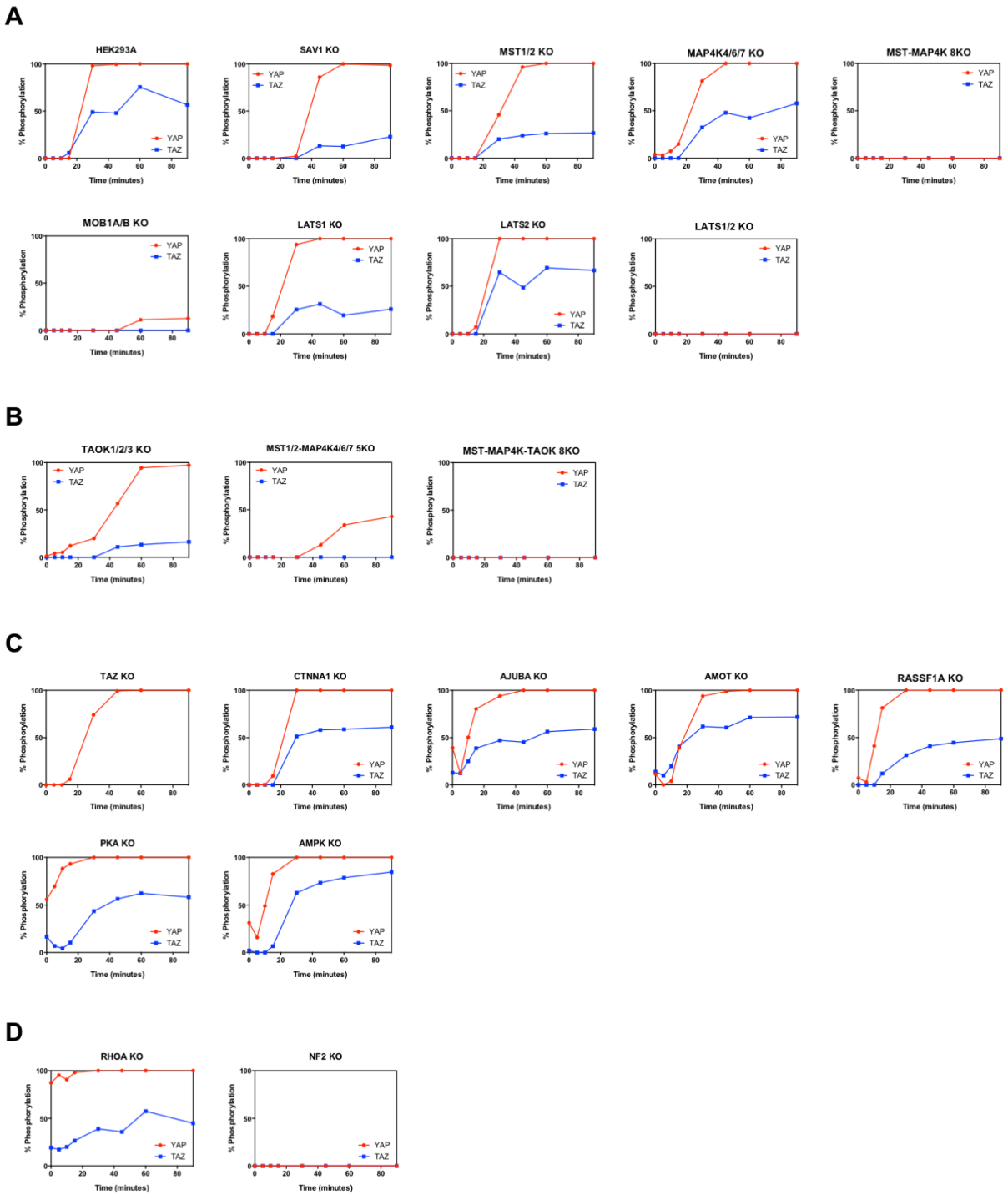


Figure S3.3. Quantification of YAP/TAZ phosphorylation.
Quantification of phosphorylated YAP and TAZ in Figure 3.3.

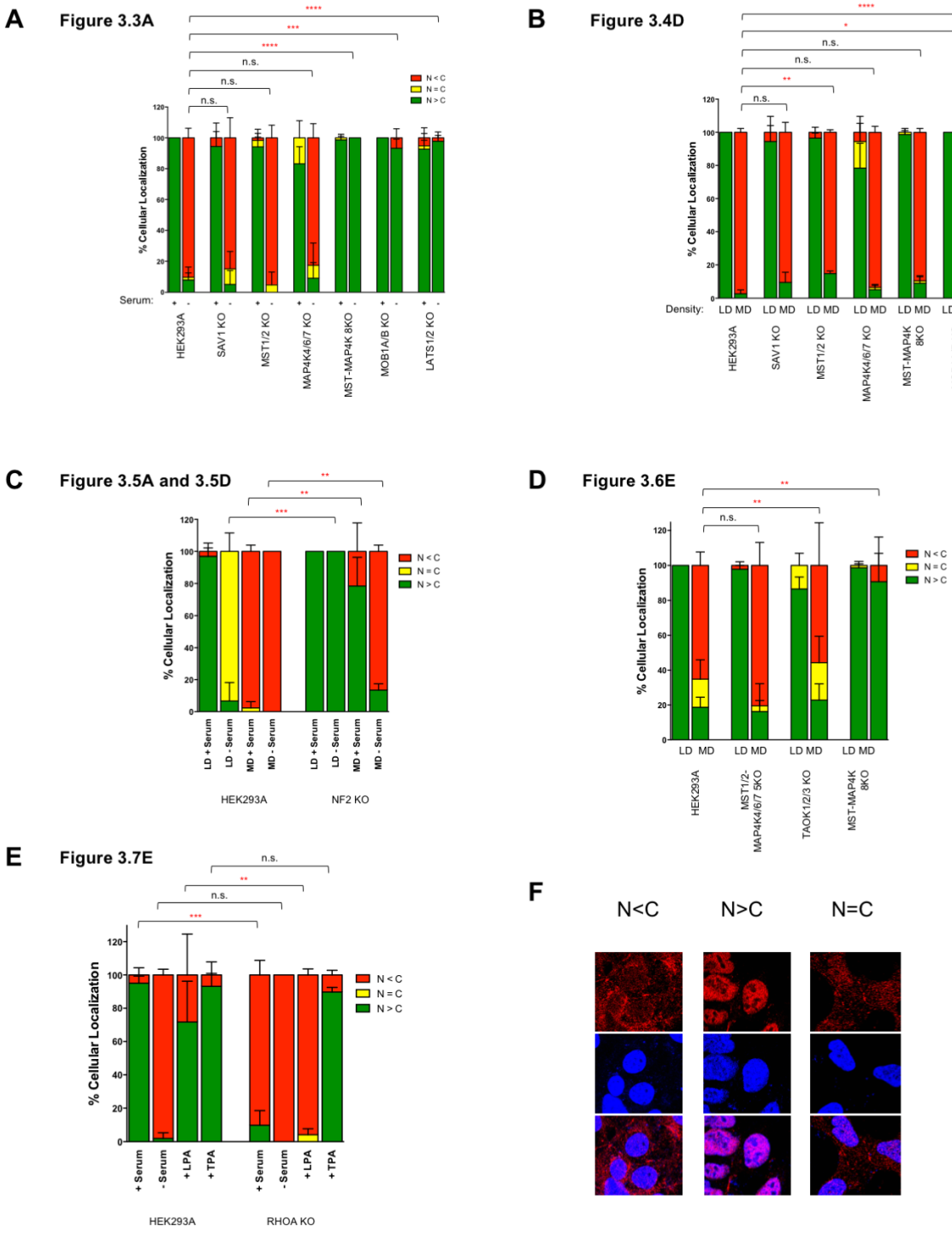


Figure S3.4. Quantification of YAP/TAZ localization.
A-E. Quantification of all immunofluorescence, based on whether YAP/TAZ are more nuclear (N>C), equally nuclear and cytoplasmic (N=C), or more cytoplasmic (C>N). Data represented as mean +/- S.D. LD indicates low density, MD indicates medium density. F. Example images showing how cells were categorized.

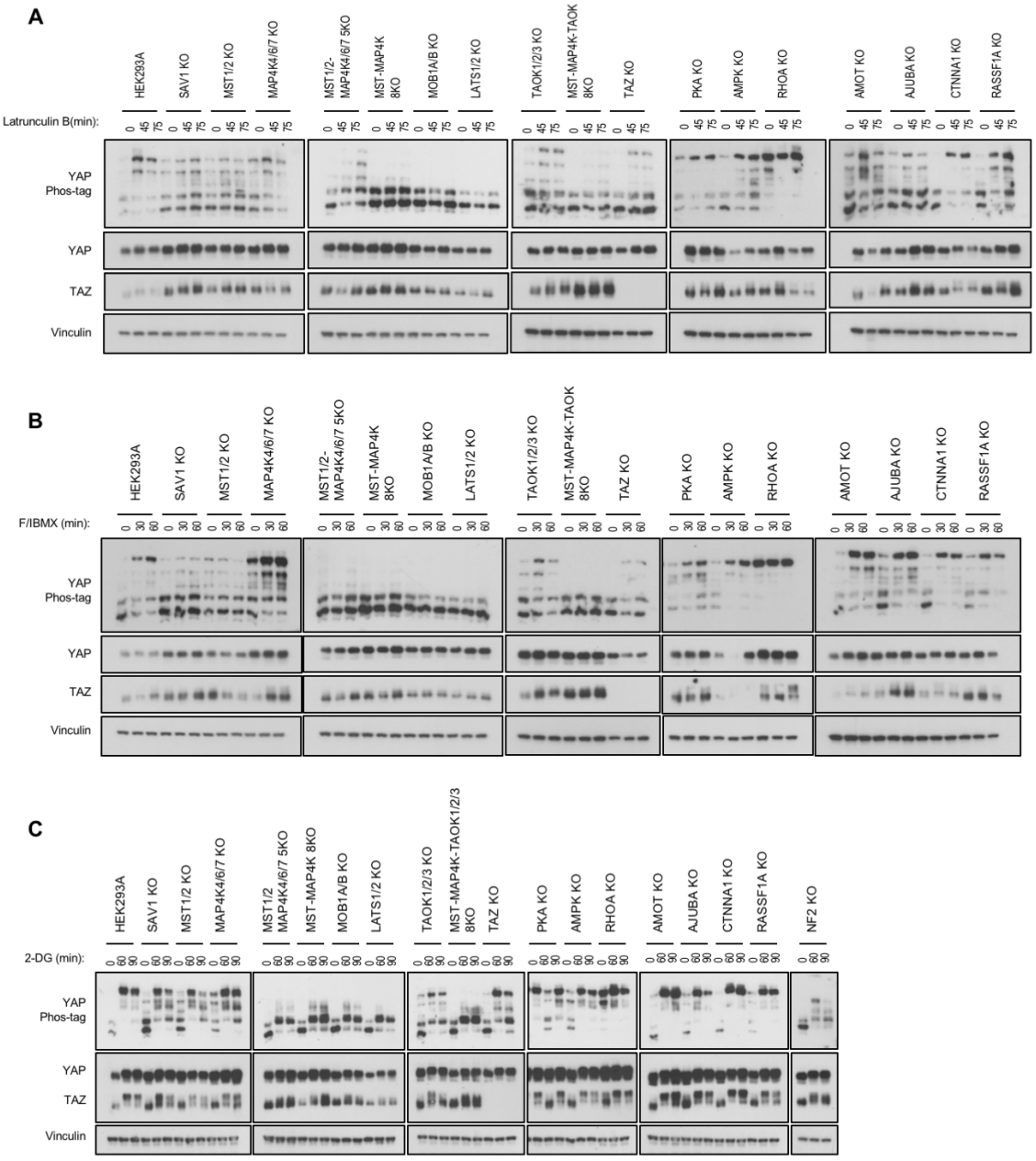


Figure S3.5. Disruption of the actin cytoskeleton, PKA activation, and cellular energy stress induce YAP phosphorylation.
A. Latrunculin B induces YAP phosphorylation by disrupting the actin cytoskeleton. Cells were treated with Latrunculin B (250 ng/ml) for 45 or 75 minutes before harvesting. B. Forskolin/IBMX induces YAP phosphorylation by activating PKA. Cells were treated with Forskolin (10 uM) and IBMX (100 uM) for 30 or 60 minutes before harvesting. C. Cellular energy stress induces YAP/TAZ phosphorylation. Cells were treated with 25 mM 2-DG in glucose-free media with 10% dialyzed serum for 60 or 90 minutes before harvesting.

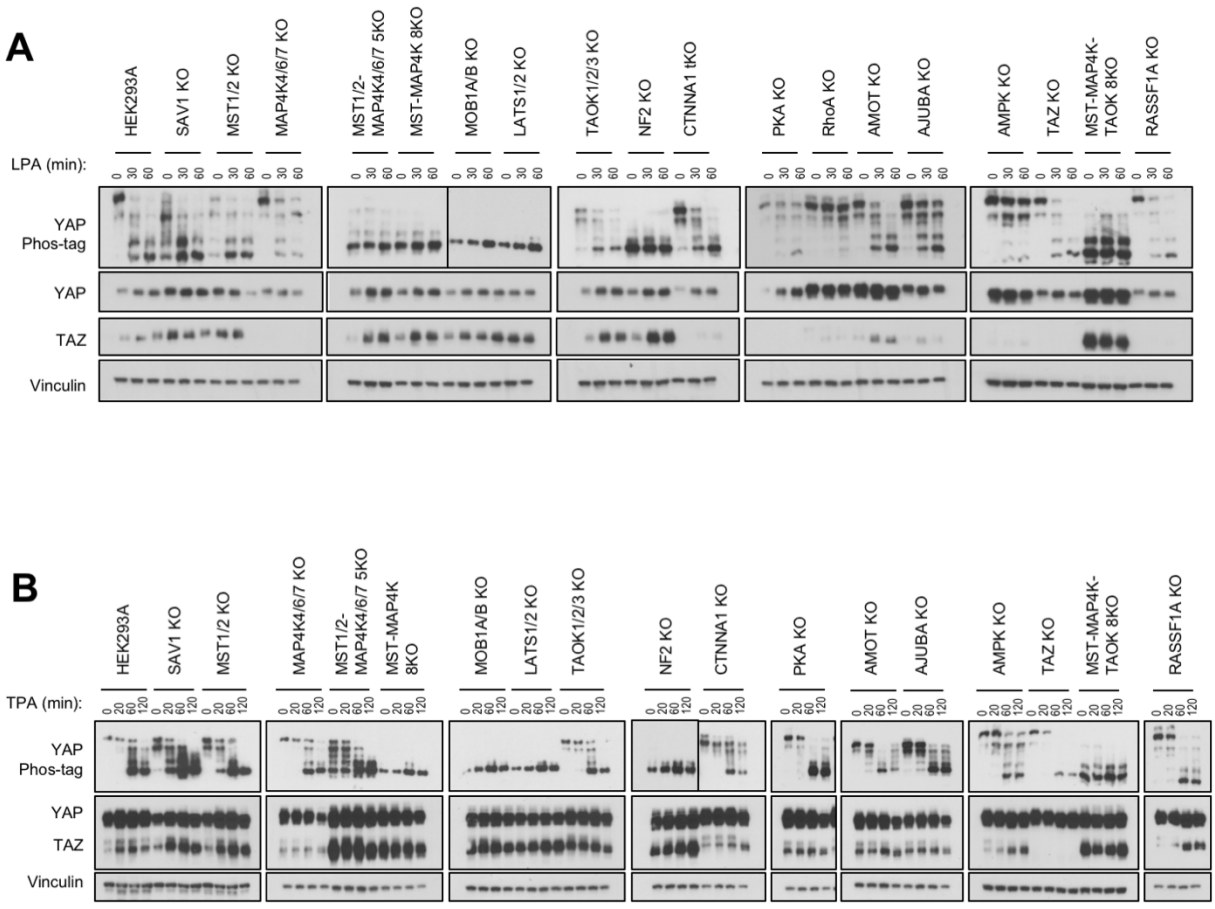


Figure S3.6. TPA and LPA induces YAP/TAZ dephosphorylation.
A. TPA induces YAP/TAZ dephosphorylation and activation. Cells were treated with TPA (5 nM) for 20, 60, or 120 minutes before harvesting. B. LPA induces YAP/TAZ dephosphorylation and activation. Cells were treated with LPA (0.5 uM) for 30 or 60 minutes before harvesting. Lines on gels indicate where irrelevant lanes were removed.

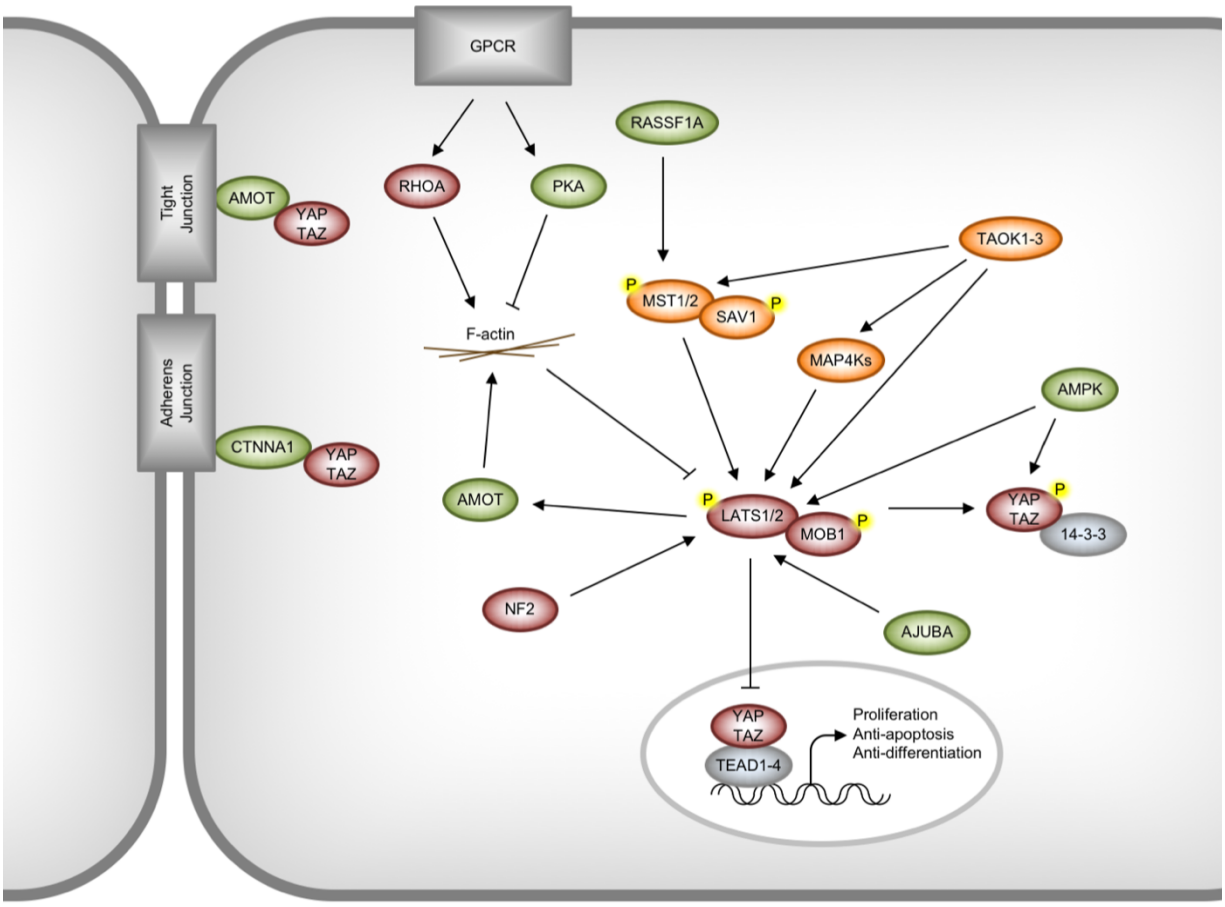


Figure S3.7. Schematic of the Hippo pathway.
Components of the Hippo pathway which are critical for proper YAP/TAZ regulation are shown in red; components of the Hippo pathway which play an important role in regulating YAP/TAZ are shown in orange; components whose deletion did not appear to affect YAP/TAZ regulation in HEK293A cells are shown in green.

Table S3.1. sgRNA sequences used in the study.

Cell Line	Clone Number	Gene	sgRNA Sequence	Reference
SAV1	N/A	SAV1	TCCAGGAGGAAGTCCTTCTC	(Park et al., 2015)
MST1/2	4-14	STK4	ATACACCGAGATATCAAGGC	(Moroishi et al., 2015)
		STK3	AGTACTCCATAACAATCCAG	
MAP4K4/6/7 KO	21-6	MAP4K4	GGGCGGAGAAATACGTTCAT	(Meng et al., 2015)
		MINK1	AGGGTCGGCATGTCAAGACG	
		TNIK	CGACTCCCGGCTCGAAGCC	
MST1/2-MAP4K4/6/7 5KO	8-12	STK4	ATACACCGAGATATCAAGGC	(Meng et al., 2015)
		STK3	AGTACTCCATAACAATCCAG	
		MAP4K4	CAGGACATGATGACCAACTC	
		MINK1	AGGGTCGGCATGTCAAGACG	
		TNIK	TTCATCCAGGCTTCGAGCCG	
MST-MAP4K 8KO	4-6	STK4	ATACACCGAGATATCAAGGC	(Meng et al., 2015)
		STK3	AGTACTCCATAACAATCCAG	
		MAP4K1	CCCATGGTAGGCCACGATGT	
		MAP4K2	GTTCGGACGTGACCGTGTCG	
		MAP4K3	CGGCGGGACAAATCGAAGCC	
		MAP4K4	GGGCGGAGAAATACGTTCAT	
		MINK1	CGGCAATGGAACCTACGGAC	
		TNIK	CGACTCCCCGGCTCGAAGCC	
MOB1A/B KO	1-2	MOB1A	CTATTCTAAAGCGTCTGTTC	
		MOB1B	TGGCAGTGGCAACCTTCG A	
LATS1/2 KO	N/A	LATS1	CGTGCAGCTCTCCGCTCTAA	(Moroishi et al., 2015)
		LATS2	TACGCTGGCACCGTAGCCCT	
TAZ	1-19	TAZ	TGTCTAGGTCCTGCGTGACG	
YAP	N/A	YAP	CATCAGATCGTGCACGTCCG	
YAP/TAZ	N/A	TAZ	TGTCTAGGTCCTGCGTGACG	(Hansen et al., 2015)
		YAP	CATCAGATCGTGCACGTCCG	
TAOK1/2/3 KO	3-5	TAOK1	TGTGCGTACCAATGAAGTGG	
		TAOK2	GCGGACCTACAAACTTCGCA	
		TAOK3	CCTTTGTACTCAATAGTATT	

Table S3.1. sgRNA sequences used in the study. (continued)

Cell Line	Clone Number	Gene	sgRNA Sequence	Reference
MST-MAP4K-TAOK 8KO	4-2	STK4	ATACACCGAGATATCAAGGC	(Meng et al., 2015)
		STK3	AGTACTCCATAACAATCCAG	
		MAP4K4	GGGCGGAGAAATACGTTCAT	
		MINK1	CGGACAGGTCGATGTCGTCC	
		TNIK	CGACTCCCCGGCTCGAAGCC	
		TAOK1	CCCAACAGTATAGAATACAA	
		TAOK2	GTACCGGGGCTGTTACCTGA	
		TAOK3	CCTTTGTACTCAATAGTATT	
RHOA	2-1	RHOA	GCTGCTCTGCAAGCTAGACG	
AMOT	N/A	AMOT	GCAAAAGGGTCTGCTGGCAC CATATGCCTCCGAGACGCGC CTTCTCTGCAAGCTGCTTGT	
AJUBA	A1	AJUBA	TTTGAGGCGCCGCGCTACGA	
CTNNA1	N/A	CTNNA1	ATGTTCAGCTTATCCACTTC	
NF2	14	NF2	GTCCATGGTGACGATCCTCA	(Moroishi et al., 2015)
AMPK	2-11	PRKAA1	TCCTGTTACAGATTGTATGC	
		PRKAA2	CGGATCTTCTTAAATAACGT	
RASSF1A	1-1	RASSF1A	AACGCGCTGCGCATCGCGCG	
PKA	A1-4	PRKACA	CAACGCCGCCGCCGCCAAGA	
		PRKCB	TAAAATCGGTCAGTTTCATC	

Table S3.2. Antibodies used in the study.

Gene	Vender	Catalog #	Recognition site
LATS1	Cell Signaling	3477	Gly180
LATS2	Cell Signaling	5888S	N-terminus
MST1	BD Biosciences	611052	AA 331-483
MST2	Abcam	ab52641	AA 1-100
MAP4K3	Cell Signaling	9613	Gly407
MAP4K4	Bethyl	A301-502A	AA 550-600
MAP4K6	Bethyl	A302-191A	AA 1-50
MAP4K7	Santa Cruz	sc-136103	AA 522-644
TAOK1	Bethyl	A300-524A	AA 400-450
TAOK2	Proteintech	21188-1-AP	AA 42-969
TAOK3	ThermoFisher	PA5-18294	CHKKDHVFIRDEAGHGD
NF2	Cell Signaling	12888	Gln470
SAV1	Cell Signaling	13301	C-terminus
P-LATS1/2 (HM)	Cell Signaling	8654	
YAP	Cell Signaling	14074	Pro435
YAP/TAZ	Santa Cruz	sc-101199	
P-YAP (S127)	Cell Signaling	4911	
MOB1	Cell Signaling	3863	N-terminus
P-MOB1 (T35)	Cell Signaling	13730	
Vinculin	BD Biosciences	V9131	
FLAG-HRP	Sigma	A8592	
HA	Cell Signaling	2367	
GST	Sigma	SAB4200237	
MYC	Cell Signaling	2278	
RHOA	Santa Cruz	sc-418	AA 120-150
AMOT	Bethyl	A303-305A	AA 1034-1084
PKA	Cell Signaling	5842	Ser326
AMPK	Cell Signaling	2532S	N-terminus
TAZ	Cell Signaling	4883S	Val386

3.6 References

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Chapter 4: Functional characterizations of the Hippo pathway effectors YAP and TAZ

4.1 Introduction

The Hippo pathway is an important regulator of tissue homeostasis and plays a critical role in development and regeneration. The core kinase cascade of the Hippo pathway consists of MST1/2 (Mammalian STE20-like 1/2), the MAP4Ks (Mitogen activate protein kinase kinase kinase kinase), and LATS1/2 (Large tumor suppressor 1/2). In response to a wide range of signals, MST1/2 and the MAP4Ks phosphorylate and activate the LATS kinases. When the core kinase cascade is activated, LATS1/2 phosphorylates and inactivates the downstream effectors of the Hippo pathway, transcriptional co-activators YAP (Yes associated protein 1) and TAZ (WW domain-containing transcription regulator 1). Because YAP and TAZ do not have their own DNA binding motifs, when dephosphorylated, they translocate to the nucleus and interact with a host of transcription factors, primarily TEAD1-4 (TEA domain family members 1-4), to induce expression of genes promoting cell growth (Piccolo et al., 2014). YAP and TAZ are not only inhibited by LATS1/2, but they are also involved in a negative feedback loop to regulate Hippo pathway kinase activity (Moroishi et al., 2015). YAP and TAZ are, if not the only, the most important downstream effectors of LATS1/2 mediating the physiological functions of the Hippo pathway.

The Hippo pathway is highly conserved, with YAP/Yorkie (the ortholog of YAP) first appearing in single cell eukaryotes (Sebe-Pedros et al., 2012). However, during evolution TAZ only appears much later, in vertebrates (Hong et al., 2005). While YAP and TAZ are generally thought to be functionally redundant, there are structural and physiological clues which suggest they may have additional, non-overlapping roles. Structurally, while YAP and TAZ share high protein sequence similarity (60%), there are significant distinctions as well (Figure 4.1A and S4.1) (Kanai et al., 2000; Santucci et al., 2015; Varelas, 2014). First, while both contain WW domains which mediate protein-protein interactions, including interactions with LATS1/2 and AMOT, YAP contains two tandem WW domains while TAZ contains only one. Additionally, YAP contains an SH3-binding motif and an N-terminal proline-rich region believed to be involved in mRNA processing, both of which are absent from TAZ. Moreover, GSK3beta has been shown to directly phosphorylate TAZ to create a second, additional phosphodegron not present in YAP, which contributes to TAZ's protein stability being much more dynamically regulated in response to phosphorylation than that of YAP (Huang et al., 2012). Finally, while all the residues necessary for YAP/TAZ interaction with TEAD1-4 are conserved, there are also differences within the TEAD binding domain. The TEAD binding domain of YAP features an extended PxxOP loop (where O is a hydrophobic residue) not found in TAZ (Chen et al., 2010; Li et al., 2010). In addition, a recent report found that TAZ-TEAD can form a hetero-tetramer complex which may affect DNA target selectivity and induce stronger expression of certain target genes (Kaan et al., 2017). Together, these structural

differences suggest there may be differences in how YAP and TAZ are regulated and how they interact with TEAD1-4 to induce gene expression.

There are physiological differences between YAP and TAZ as well. YAP knockout mice are embryonic lethal at E8.5 due to severe developmental defects (Morin-Kensicki et al., 2006). Conversely, TAZ knockout is only partially lethal, with 1/5 of the mice being viable, although they develop renal cysts and lung emphysema (Hossain et al., 2007; Makita et al., 2008; Tian et al., 2007). Thus, YAP and TAZ are not completely redundant because TAZ is unable to compensate for the loss of YAP. What is not clear, however, is whether this is due to differences in tissue distribution and expression, or actual regulatory or transcriptional differences between the two genes.

Therefore, an open question in Hippo biology is what are the differences in the transcriptional profiles of YAP and TAZ, and what are the downstream physiological implications of these differences. To this end, we used CRISPR/Cas9 to create YAP or TAZ single knockout, and LATS1/2 and YAP/TAZ double knockout cell lines and performed a wide array of assays and comparisons to delineate any differences between YAP and TAZ and to better characterize the consequences of dysregulated Hippo pathway signaling.

4.2 Results

Comparison of YAP and TAZ in TEAD interaction and target gene expression

We used CRISPR/Cas9 to create LATS1/2 KO (knockout), YAP KO, TAZ KO, and YAP/TAZ KO cell lines in HEK293A cells (Plouffe et al., 2016). In addition to sequencing, we also performed siRNA and rescue experiments to ensure that our knockouts were specific (Figure S4.2A-B). The first question we addressed was how the loss of YAP or TAZ affects activation and regulation of the upstream Hippo pathway kinase cascade. Serum is one of the most potent activators of YAP/TAZ; LPA (lysophosphatidic acid) present in serum activates GPCRs (G-protein coupled receptors) to inactivate the Hippo pathway, resulting in dephosphorylated, nuclear, and transcriptionally active YAP and TAZ. In the presence of serum, YAP and TAZ are dephosphorylated at S127 and nuclear in all cell lines (Figure 4.1B and 4.1C). Following starvation, YAP and TAZ are phosphorylated in all cell lines except the LATS1/2 KO cells; this is expected because LATS1/2 are the primary kinases for YAP/TAZ in response to starvation, so following their deletion the cell is unable to compensate to inactivate YAP/TAZ. TAZ protein levels were significantly elevated in the LATS1/2 KO cells, supporting the notion that TAZ is destabilized by LATS-dependent phosphorylation. However, expression and activation of upstream components LATS1/2 and MOB1A/B appear unaffected by loss of YAP, TAZ, or both YAP/TAZ.

Moreover, the same trends are evident when we look at downstream transcriptional activity. YAP/TAZ target genes CTGF (Connective tissue growth factor) and CYR61 (Cysteine rich angiogenic inducer 61) are both induced, while LGR5 (Leucine rich repeat containing G-protein coupled receptor 5) is repressed by active YAP and TAZ. Following serum stimulation, CTGF and CYR61 expression was

induced in all cell lines except the YAP/TAZ KO cell line (Figure 4.1D and 4.1E), indicating that CTGF and CYR61 induction is YAP/TAZ-dependent. Deleting YAP had a more dramatic effect on CTGF and, to a lesser extent, on CYR61 expression than that of TAZ, as induction in the YAP KO cell line was significantly reduced relative to the wild-type and TAZ KO cell lines. LGR5 expression was increased in all the YAP KO, TAZ KO, and YAP/TAZ KO cell lines compared to the wild-type and LATS1/2 KO cell lines, and consistently, was strongly repressed in the LATS1/2 KO cells (Figure 4.1F). These data suggest that both YAP and TAZ are involved in repression of LGR5, such that deleting either YAP or TAZ is sufficient to cause increased LGR5 expression, whereas YAP is more important than TAZ in the induction of CTGF and CYR61.

The most noticeable structural difference between YAP and TAZ is the WW domains, of which YAP has two but TAZ only has one (Figure 4.1A). We questioned whether these structural differences may yield some insight into potential differences in how YAP and TAZ are regulated or how they interact with TEAD to induce transcription. We deleted each of the WW domains in YAP and assayed the effect on YAP activity by comparing CTGF induction after expressing the YAP mutants in YAP/TAZ KO cells. We found that the single deletion of a WW domain had little effect, while deletion of both WW domains partially compromised CTGF induction (Figure S4.3A-C). Meanwhile, phosphorylation is the major mechanism for YAP/TAZ regulation. We created point mutations for each of the conserved serine residues, except those in the C-terminal transactivation domain, to determine which potential phosphorylation sites are most important for YAP transcriptional activity (Figure

S4.3A-C). Our goal was that, if we identified serines critical for YAP transcriptional activity, then we could also determine whether they are critical for TAZ transcriptional activity, which may yield some insight into how YAP/TAZ are differentially regulated. However, none of these mutations had a significant effect on downstream target gene (CTGF) expression relative to the wild-type YAP with the exception of S94A (which prevents YAP-TEAD binding) and S127A (which prevents YAP-14-3-3 binding), both of which are already well established and conserved in TAZ (Figure S4.3C). These observations support S94 and S127 as key regulatory phosphorylation sites and highlight the importance of interaction with the TEAD transcription factors and cytoplasmic localization by 14-3-3 binding in controlling YAP function.

TEADs are the main transcription factors and nuclear binding partners of YAP/TAZ. When TEADs are not in the nucleus, even un-phosphorylated YAP cannot be localized in the nucleus (Lin et al., 2017). Any physiological or transcriptional differences between YAP and TAZ could be due to differences in their binding affinities or interactions with TEADs. Thus, we examined the interaction between TEADs and YAP and TAZ by co-immunoprecipitation. Our data showed that YAP or TAZ displayed similar interactions with TEAD1, TEAD2, and TEAD4 (Figure S4.3D). We did not include TEAD3 because it is lowly expressed in HEK293A cells (Figure S4.3E). This indicates that the differential effect of YAP and TAZ on gene expression is unlikely due to a difference in TEAD binding because YAP and TAZ interacted similarly with each of the TEADs. Interestingly, protein levels of TEAD1, TEAD2, and TEAD4 were all decreased in the YAP KO, TAZ KO, and YAP/TAZ KO cells (Figure

S4.3F), suggesting that YAP/TAZ and proper TEAD expression are linked, although it may not be a direct regulation. Nevertheless, this supports an interdependent relationship between YAP/TAZ and TEADs.

Effect of YAP and TAZ on cell growth and size

First, we observed that dysregulation of the Hippo pathway affected cell spreading (Figure 4.2A-B). When plated on plastic, the LATS1/2 KO cells showed increased cell spreading, while the YAP KO and YAP/TAZ KO cells were significantly smaller. The TAZ KO cells showed no difference from the wild-type cells. This was also observed when cells were plated on poly-lysine (Figure 4.2C).

Because cell spreading is only one measure of cell size, we also used FACS (Fluorescence activated cell sorting) to compare cell volume and granularity. Consistent with what we observed with cell spreading, the LATS1/2 KO cells exhibited a significant increase in volume and granularity relative to the wild-type cells, while the YAP KO and YAP/TAZ KO cells showed significant decreases in both volume and granularity (Figure 4.2D-F). Consistent with what we observed transcriptionally (Figure 4.1D-E), deleting YAP had a greater impact on cell size than did deleting TAZ.

While it is not clear what might account for the changes in granularity, one potential explanation may be differences in mitochondrial number; if the LATS1/2 KO cells have increased mitochondria, that might also explain their increased growth capacity. However, no differences in the relative mitochondria content of the different

cell lines were observed when comparing mitochondrial DNA levels (Figure 4.2G and S4.4A).

Effect of YAP and TAZ on cell physiology

To compare the physiological consequences of dysregulated YAP and TAZ, we first looked at glucose uptake and metabolism. Cells were plated and given fresh medium for 6 hours, after which the remaining glucose levels in the culture media were measured. The LATS1/2 KO cells took up glucose at a faster rate and had lower remaining glucose levels in the culture media than the wild-type cells, while the YAP KO and YAP/TAZ KO cells' glucose uptake was reduced (Figure 4.3A). Interestingly, the TAZ KO cells were not significantly different from the wild-type cells.

Next, we compared rates of cell proliferation. As expected, the LATS1/2 KO cells with constitutively active YAP and TAZ proliferated at a rate slightly faster than the wild-type cells (Figure 4.3B). The modest effect of LATS1/2 KO on cell growth is likely due to the fact that, under normal growth conditions (low cell density and the presence of abundant serum and glucose), LATS activity is repressed. The TAZ KO cells clustered closely with the wild-type cells, while the YAP KO and YAP/TAZ KO cells showed a dramatically decreased rate of proliferation. When we performed cell cycle analysis of these cells in complete growth conditions, there were no significant differences between any of the cell lines (Figure 4.3C), indicating that the YAP KO and YAP/TAZ KO cells proliferate slower overall but are not specifically impaired at any one stage of the cell cycle.

The migratory capacity of each cell line was also tested in a trans-well migration assay utilizing an 8 um PET pore membrane. 8 hours after plating, the LATS1/2 KO cells showed significantly increased migratory capacity relative to the wild-type cells, while the YAP KO and YAP/TAZ KO cells both showed decreased migratory capacity (Figure 4.3D and 4.3E).

Finally, we tested the migratory potential of each cell line using a scratch assay. Although the LATS1/2 KO cells showed increased migration over 48 hours, there were no differences between the wild-type, YAP KO, TAZ KO, and YAP/TAZ KO cell lines (Figure 4.3F and S4.4B). To ensure that the wound closure we observed is due to migration and not cell proliferation, cells were maintained in serum-free media. Under prolonged starvation conditions, YAP and TAZ are both phosphorylated and inactivated, which may explain why the wild-type, YAP KO, TAZ KO, and YAP/TAZ KO cells behaved similarly.

Effect of YAP and TAZ on cell signaling

We next compared whether there were any kinetic differences between the regulation of YAP and TAZ. It is possible that regulation of one is primarily responsible for inducing immediate response genes, while regulation of the other is responsible for inducing a second set of slower responding genes. However, when we performed a time-course of serum stimulation on each of these cell lines, YAP and TAZ activation appeared unaffected in the knockout cell lines and both showed dephosphorylation beginning around 30 minutes following stimulation (Figure S4.4C). Moreover, this was consistent when we compared YAP and TAZ nuclear localization

(Figure 4.4A).However, one difference we did note was that, following 60 minutes of serum stimulation, YAP became completely dephosphorylated at S127 (Figure S4.4C). Conversely, under the same conditions, TAZ was only partially dephosphorylated based on phos-tag gel analysis. Even in the LATS1/2 KO cells, when YAP was completely dephosphorylated, almost 50% of TAZ remained highly phosphorylated. We have yet to identify any conditions in which TAZ is completely dephosphorylated. To confirm whether this upper band was due to phosphorylation or some other post-translational modification, we treated the lysates with lambda phosphatase (Figure 4.4B). Following treatment, the upper band of TAZ disappeared, demonstrating that TAZ is not completely dephosphorylated in response to serum stimulation. This raises the possibility that there are additional LATS1/2-independent kinases that specifically phosphorylate TAZ but not YAP. However, under stimulation conditions, TAZ is largely nuclear, suggesting that this LATS1/2-independent phosphorylation does not affect TAZ protein localization. Thus, it is unclear whether this remaining phosphorylation has any effect on TAZ co-transcriptional activity, although it appears that it is not a compensatory mechanism because even following loss of YAP, TAZ does not become ‘more fully’ activated in response.

We next investigated whether the physiological differences we observed between YAP and TAZ could be due to differences in protein expression. Although mRNA expression of TAZ is slightly higher than that of YAP by RNA-seq, this does not always translate into corresponding differences in protein expression (Figure 4.5A). Because TAZ has two phosphodegrons, its protein stability is much more dynamically regulated than that of YAP, which only has one phosphodegron. For

instance, following 6 hours of serum starvation, TAZ protein levels decreased much more dramatically than that of YAP (Figure 4.5B). To compare the relative protein levels of endogenous YAP and TAZ, we first needed to determine the relative sensitivity of the YAP/TAZ antibody that recognizes both YAP and TAZ. To calibrate the YAP/TAZ antibody, we transfected HA-YAP and HA-TAZ and compared the expression levels as detected with the HA antibody, which should be equally sensitive to both HA-YAP and HA-TAZ. The HA-YAP and HA-TAZ in the same samples were then also detected with the YAP/TAZ antibody (Figure 4.5C). Based on the above analyses, our data indicate that the YAP/TAZ antibody detects both YAP and TAZ with similar sensitivity. Thus, according to the Western blot signals of endogenous YAP and TAZ detected by the YAP/TAZ antibody, we concluded that the endogenous YAP protein levels are more than twice that of TAZ in HEK293A cells under normal growth conditions. Therefore, even though TAZ has higher mRNA expression relative to YAP, its lower protein stability may contribute to the lower protein levels. Therefore, the higher YAP protein levels may explain why deleting YAP has a greater effect on cell size and physiology than deleting TAZ.

To confirm that the transcriptional differences we observed in CTGF induction is primarily due to changes in protein expression, we then transfected varying amounts of HA-YAP and HA-TAZ in the YAP/TAZ KO cells, along with a CTGF-luciferase reporter (Figure 4.5D-E). Induction of the luciferase reporter was equivalent between HA-YAP and HA-TAZ when they were equally expressed, suggesting that the biggest difference of endogenous YAP and TAZ in their ability to

induce CTGF and potentially other downstream target genes is due to their protein expression.

In addition, we also wanted to determine whether regulation of YAP/TAZ phosphorylation and this YAP/TAZ-dependent mechanism of CTGF induction was unique to HEK293A cells, or whether it is more broadly applicable. We also generated knockouts in HeLa and MCF7 epithelial cells (Figure 4.5F and S4.4D). In HeLa cells, knockout of YAP/TAZ was sufficient to completely ablate CTGF expression. Furthermore, in the MCF7 cells, inactivation of LATS1/2 was sufficient to result in dephosphorylated YAP and increased CTGF expression, even under starvation conditions, demonstrating that these mechanisms are more broadly conserved.

Finally, we also tested the possibility that YAP/TAZ may play an important role in response to different environmental cues or cellular stresses. To this end, we first compared YAP and TAZ phosphorylation in response to a variety of stresses, including glucose starvation, actin disruption by Latrunculin B, activation of PKA by Forskolin/IBMX (3-isobutyl-1-methylxanthine), and exposure to cerivastatin (HMG-CoA reductase inhibitor) (Figure S4.5A). We observed similar regulation of YAP and TAZ phosphorylation in the different cell lines in response to the various treatments. Next, we compared the wild-type, LATS1/2 KO, and YAP/TAZ KO cells in response to long-term exposure to several types of stress, including exposure to LPS (lipopolysaccharides), toxin (Streptolysan O), dobutamine (b1 receptor agonist), rapamycin (inhibits mTOR), hypoxia, serum starvation, osmotic stress, ER stress, phosphatase inhibitors, cerivastatin, apoptosis, DNA damage, AMPK inhibitors, and

glucose starvation (Figure S4.5B). However, we did not see any significant differences in long-term survival between any of the cell lines under these conditions, indicating that YAP/TAZ do not play an important role in response to these specific stresses.

Effect of YAP and TAZ on transcription

Because serum activates many other pathways in addition to YAP and TAZ, we focused on LPA, which is the most potent YAP/TAZ activator in serum (Yu et al., 2012). Using a low concentration of LPA, we performed a time-course in each of the cell lines to determine whether there were differences in their transcriptional responses to LPA stimulation (Figure 4.6A). Similar to what we saw following serum stimulation (Figure 4.1D-F), deleting YAP had a greater effect on CTGF induction than deletion of TAZ. To further delineate whether there are other differences in the transcriptional programs of YAP and TAZ, we performed RNA-seq for the wild-type, YAP KO, TAZ KO, and YAP/TAZ KO cell lines under starvation and LPA stimulation conditions (100 nM, 90 minutes). In doing so, our goal was two-fold: first, we wanted to identify bona fide YAP/TAZ target genes; second, we wanted to identify any differences between the transcriptional programs of YAP and TAZ.

Surprisingly, there were very few genes that were statistically significant and showed greater than a 2-fold change in expression following stimulation (Figure 4.6B). This is probably due to the fact that the LPA stimulus was intentionally weak to identify YAP/TAZ-dependent responses while hopefully minimizing any secondary responses. The two genes that showed the biggest induction in the wild-type, YAP

KO, and TAZ KO cell lines were CTGF and CYR61. Additionally, they showed no induction in the YAP/TAZ KO cells, confirming that induction of CTGF and CYR61 expression is dependent on both YAP and TAZ.

To summarize some of the genes which showed at least a 1.5-fold induction, there were several genes which showed induction in the wild-type, YAP KO, and TAZ KO cell lines but not the YAP/TAZ KO cell line (Figure 4.6C). These genes included CTGF, CYR61, ATF3 (Activating transcription factor 3), and FILIP1L (Filamin A-interacting protein 1-like). Induction of all of these in response to LPA stimulation was dependent on both YAP and TAZ; both YAP and TAZ were involved because they compensated for the loss of the other, as LPA-induced expression was seen in the single knockouts but not the YAP/TAZ KO cells.

There were also genes such as AMOTL2 (Angiomotin like 2) and FOSL1 (Fos-like antigen 1) which were induced in the wild-type and TAZ KO cell lines, but not in the YAP KO or YAP/TAZ KO cell lines (Figure 4.6D). Induction of these genes was YAP-dependent. AMOTL2 is part of the AMOT (Angiomotin) family of proteins, which induces LATS2 phosphorylation and YAP cytoplasmic sequestration. The fact that AMOTL2 was induced in the wild-type and TAZ KO cells following LPA stimulation could indicate a potential YAP-dependent feedback mechanism.

Amongst the genes that did show at least a 1.5-fold induction in the wild-type cells following LPA stimulation, we found that a number of them were immediate-early response genes, and several of them were compromised in the YAP KO and YAP/TAZ KO cell lines (Figure 4.6E) (Tullai et al., 2007). Indeed, a GO pathway analysis of these genes revealed that many of them are transcription factors or

growth factors, supporting an important role for YAP in mediating induction of immediate-early response genes (Figure S4.6A). Overall, deleting YAP had a greater effect on downstream transcription than deleting TAZ. However, before the LPA stimulation, cells were starved overnight, and based on what we observed previously (Figure 4.5B), TAZ protein levels were probably significantly reduced at this point due to degradation such that this might explain why there does not appear to be much difference between the starved wild-type cells and the TAZ KO cells.

One important caveat to note is that baseline expression for many genes may already be significantly altered following deletion of either YAP or TAZ. To compare how deleting YAP or TAZ affects the baseline transcriptional states of the cells, we compared the YAP KO, TAZ KO, and YAP/TAZ KO cell lines relative to the wild-type cells under baseline starvation conditions (Figure S4.6B). In total, the YAP KO cell line had 294 differentially expressed genes, the TAZ KO cell line had 202, and the YAP/TAZ KO cell line had 324 compared to the wild-type cells. This suggests that deleting YAP or TAZ alone is sufficient to cause widespread changes in the transcriptional landscapes of the cells. Of the 294 differentially expressed genes in the YAP KO cells, 81% of them were similarly differentially expressed in the YAP/TAZ KO cells. For the TAZ KO cells, this percentage drops to 49%. This suggests that deleting YAP has a greater effect on the transcriptional landscape of the cell than deleting TAZ. Thus, changes in induction between the cell lines may also be due to differences in their basal transcriptional levels.

4.3 Discussion

The Hippo pathway plays an important role in regulating cell growth, proliferation, and tissue homeostasis. Thus, it is not surprising that dysregulation of the Hippo pathway results in significant cellular changes and has been implicated in many human diseases, particularly cancer (Plouffe et al., 2015). To better characterize the cellular effects of dysregulated Hippo signaling, we analyzed the physiological consequences of inactivating the key effectors of the Hippo pathway, YAP and TAZ.

Through comparing the LATS1/2 KO cells, in which YAP/TAZ are constitutively-active, and the YAP/TAZ KO cells, we were able to clearly delineate the consequences of dysregulated YAP/TAZ signaling. First, it is clear that YAP/TAZ are master regulators for a variety of cellular processes, including cell spreading, controlling cell volume, glucose uptake and metabolism, cell proliferation, migration, and downstream gene expression, and that dysregulation of YAP/TAZ alone can have significant consequences on the cell. Especially given that several of these phenotypes, particularly control of cell proliferation, cell size, and migration, are correlated with cancer stem cell-like properties and metastasis, these findings reaffirm the oncogenic potential of YAP/TAZ and their attractiveness as therapeutic targets. When comparing the differences between cell lines, the YAP KO cells clustered more closely with the YAP/TAZ KO cells with regards to many of these phenotypes; thus, targeting YAP may be more efficacious than targeting TAZ. Although TAZ does have make contribution, as attested to by the differences

between the YAP KO and YAP/TAZ KO cells, it appears that loss of TAZ is largely compensated for by the presence of YAP. Biochemically, the nuclear translocation and TEAD binding of YAP and TAZ are similar. However, YAP protein levels are significantly higher than that of TAZ in HEK293 cells; therefore, we posit that the difference in YAP and TAZ protein levels may contribute to their functional differences in these cells. Interestingly, we found that there remains significant LATS1/2-independent phosphorylation of TAZ. While it remains unclear under which conditions TAZ may become completely dephosphorylated or whether this phosphorylation plays any role in regulating TAZ protein stability or transcriptional activity, this raises the possibility that TAZ may have some context-specific or even cell-type dependent activity, or that there may still be some mechanisms by which regulation of YAP and TAZ diverge. Further work is warranted.

Furthermore, even though it is clear that inactivating YAP in HEK293A cells has a greater effect than inactivating TAZ, it is also evident that YAP and TAZ are not completely functionally redundant. There is evidence for this from the RNA-seq, with genes such as AMOTL2 and FOSL1 whose inductions are YAP-dependent but not TAZ-dependent. This again raises the possibility that, although there is certainly significant overlap, YAP and TAZ may induce slightly different transcriptional profiles. This may be dependent on the type and strength of the stimuli. Therefore, it will be interesting to perform additional comparisons across other types of stimuli and cell types to better understand what the differences might be, as well as even coming to a better grasp as to the significance of why TAZ only appears in vertebrates and what evolutionary role it might play.

Finally, as previously reported, YAP, TAZ, and TEAD1-4 are already nuclear and transcriptionally active in the LATS1/2 KO cells, even under starvation conditions. However, serum stimulation was still able to induce CTGF and CYR61 expression in these cells (Figure 4.1D-E). This additional induction could be due to activation of other transcription factors, such as AP-1 which has been reported to synergize with TEAD1-4 in promoting gene expression (Liu et al., 2016; Zanconato et al., 2015). This highlights the observation that regulation of TEAD1-4 or other co-transcription factors such as AP-1, have a major role in amplifying the downstream target gene expression of active YAP/TAZ. Nevertheless, this induction is completely dependent on YAP/TAZ because no such induction is seen in the YAP/TAZ KO cells.

In conclusion, we have analyzed the cellular and transcriptional consequences of inactivating YAP and TAZ. One note of caution is that our studies were conducted primarily in HEK293A cells, and it is entirely possible that YAP and TAZ may have tissue-specific roles during development and regeneration due to differences in signaling or distribution. Other potential differences between YAP and TAZ may involve tissue-specific or cell-type specific transcription factor binding partners, or other mechanisms which regulate YAP/TAZ protein stability and expression. This may explain some of the differences we see between the YAP and TAZ knockout mouse models. However, because YAP knockout mice are embryonic lethal, and several other YAP conditional knockout models are also lethal, it complicates comparing the consequences of inactivating YAP and TAZ in vivo. Additionally, many in vivo studies only focus on YAP or TAZ, but do not compare the two. For example, while lung-specific conditional knockout models have been generated for many Hippo

pathway components, lung-specific TAZ knockout mice have not been studied (Dai et al., 2017). Our goal was to more comprehensively characterize some of the physiological differences between YAP and TAZ in vitro in an attempt to shed greater insight on this topic. While more work remains, this systematic functional analysis of YAP and TAZ provides a useful resource in the quest to better understand the commonalities and differences between YAP and TAZ and the downstream effects of dysregulated Hippo signaling.

4.4 Experimental Procedures

Generating Cell lines

pSpCas9(BB)-2A-Puro (PX459; Addgene plasmid #48139) was a gift from Dr. Feng Zhang (Shalem et al., 2014). Gene-specific sgRNAs were designed and cloned as previously described (Plouffe et al., 2016). HEK293A cells were transfected using PolyJet Transfection Reagent, selected with puromycin for 3 days, and single-cell sorted by FACS into 96-well plates. Single clones were expanded and screened by Western blot and confirmed by sequencing.

Cell culture

HEK293A cells were grown in DMEM with 10% FBS (Fetal bovine serum) and 1% P/S (Penicillin/streptomycin). Cells were plated at $1.5x10^5$ cells per well into 6-well plates, and were serum starved overnight before serum or LPA (100 nM, 90 min) stimulation.

Western Blot

Western blots were performed as previously described (Plouffe et al., 2016). 7.5% phos-tag gels were used to compare YAP and TAZ phosphorylation.

FACS Sorting

Cells were fixed in cold 70% ethanol overnight. Cells were then washed twice with PBS, treated with 10 ug/ml RNAse at 37-C for 30 minutes, and stained with 50 ug/ml PI.

Glucose Measurements

Cells were plated at $8x10^5$ cells per well into 6-well plates. Once the cells adhered to the dish, the culture media was replaced with 1 ml of fresh DMEM with 10% FBS and 1% P/S for 6 hours. Glucose levels were measured using a FreeStyle Precision Neo glucose monitoring system.

Migration Assay

For the migration assay, we used Falcon Cell culture inserts (transparent PET membrane, 24-well, 8.0 um pore size). The bottom of each insert was coated with poly-lysine for 1 hour at 37-C, after which the poly-lysine was removed and the insert allowed to air dry at room temperature for 1 hour. $5x10^4$ cells were plated inside the insert in serum-free culture media, while the bottom of the well was filled with

complete culture media. Cells were allowed to migrate for 8 hours, after which they were fixed and stained with 0.5% crystal violet in methanol.

Scratch Assay

6-well plates were coated with poly-lysine for 1 hour at room temperature, before being washed three times with PBS. Cells were then plated at $1.5x10^6$ cells per well into 6-well plates. Once the cells adhered to the dish, the culture media was replaced with serum-free media and the scratch was made using a plastic pipette tip.

Immunofluorescence

Immunofluorescence was performed as previously described (Plouffe et al., 2016).

Lambda Phosphatase treatment

Cells were lysed in mild lysis buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1% NP-40, protease inhibitor cocktail). Then 10 ul NEB buffer, 10 ul McCl, and 2 ul lambda phosphatase was added and incubated at 37-C for 1 hour. Reaction was terminated by adding 4x loading buffer (200 mM Tris pH 6.8, 8% SDS, 0.1% Bromophenol blue, 40% glycerol, 20% 2-Mercaptoethanol). Samples were then boiled for 5 minutes and loaded directly onto the gel for Western blot analysis.

RNA-seq

Total RNA was extracted and used to prepare libraries using Illumina TruSeq Stranded mRNA Library Prep Kit Set A (Illumina, RS-122-2101) or Set B (Illumina, RS-122-2102). The libraries were sequenced using Illumina HiSeq 4000 (single end 50bp).

Sequenced reads were aligned to the hg19 reference genome using STAR (Dobin et al., 2013). Only uniquely mapped reads were kept for further analysis. The number of reads for each gene were counted using htseq-count (Anders et al., 2015) based on the Gencode human annotation release 24. Differentially-expressed genes were identified using DESeq2 (Love et al., 2014). Specifically, genes with adjusted p-value < 0.1 were considered as differentially expressed. Biological triplicates were used.

Luciferase Assay

Luciferase reporter assay was performed as previously described (Mo et al., 2015). Briefly, cells were transfected with a pGL3-CTGF-Luc reporter. Luciferase activity was assayed using the Neolite Reporter Gene Assay System (PerkinElmer) according to the manufacturer's instructions.

Statistical Analysis

Where indicated, experiments were repeated at least three times and statistical analysis was performed using unpaired t tests. ns, $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

4.5 Acknowledgments

Chapter 4, in full, has been submitted for publication of the material as it may appear in the Journal of Biological Chemistry; Plouffe, S.W., Lin, K.C., Moore, J.L., Tan, F.E., Ye, Y., Qui, Y., Ren, B., Guan, K.L. ASBMB Press, 2018. The dissertation author was the primary investigator and author of this paper.

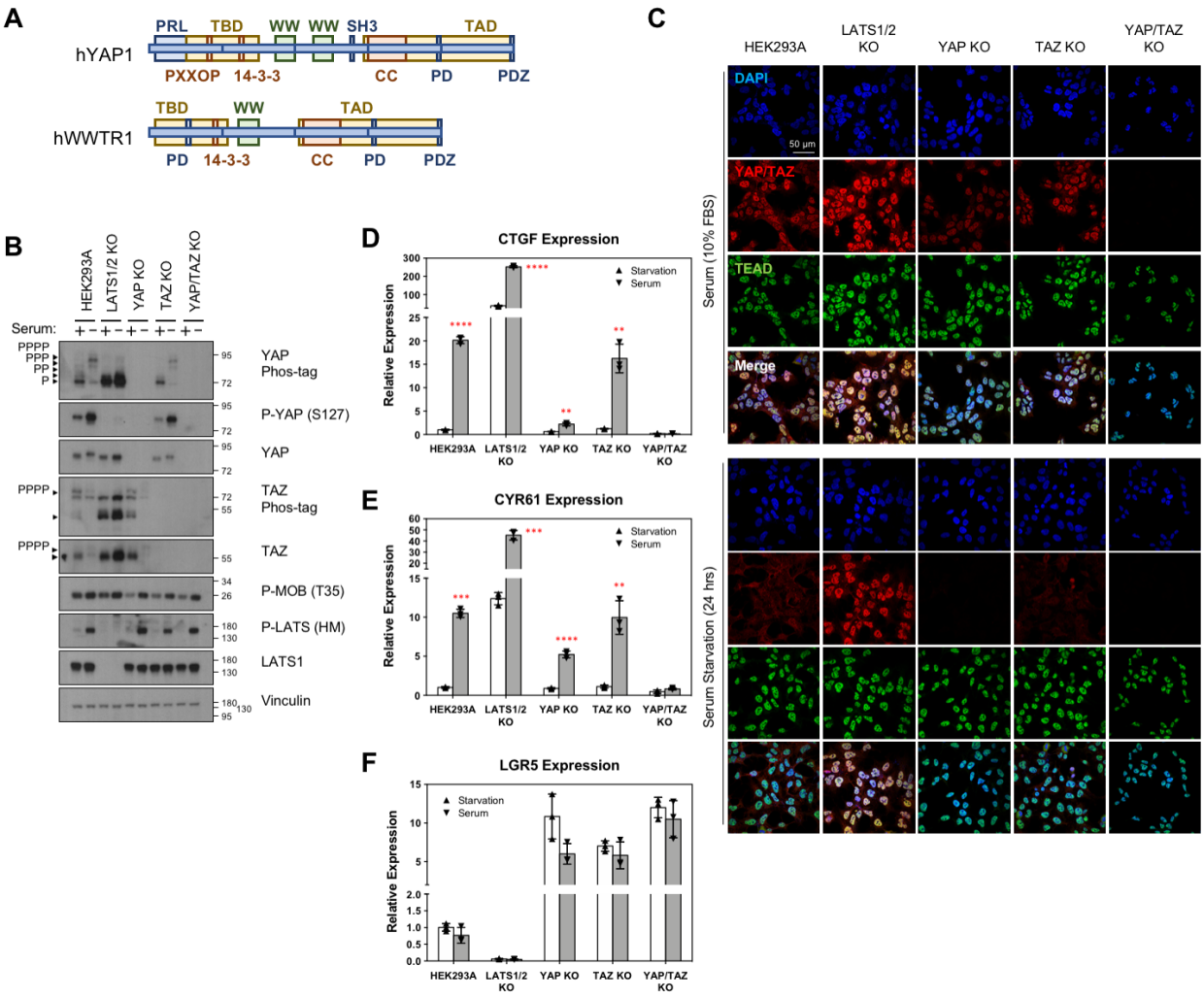
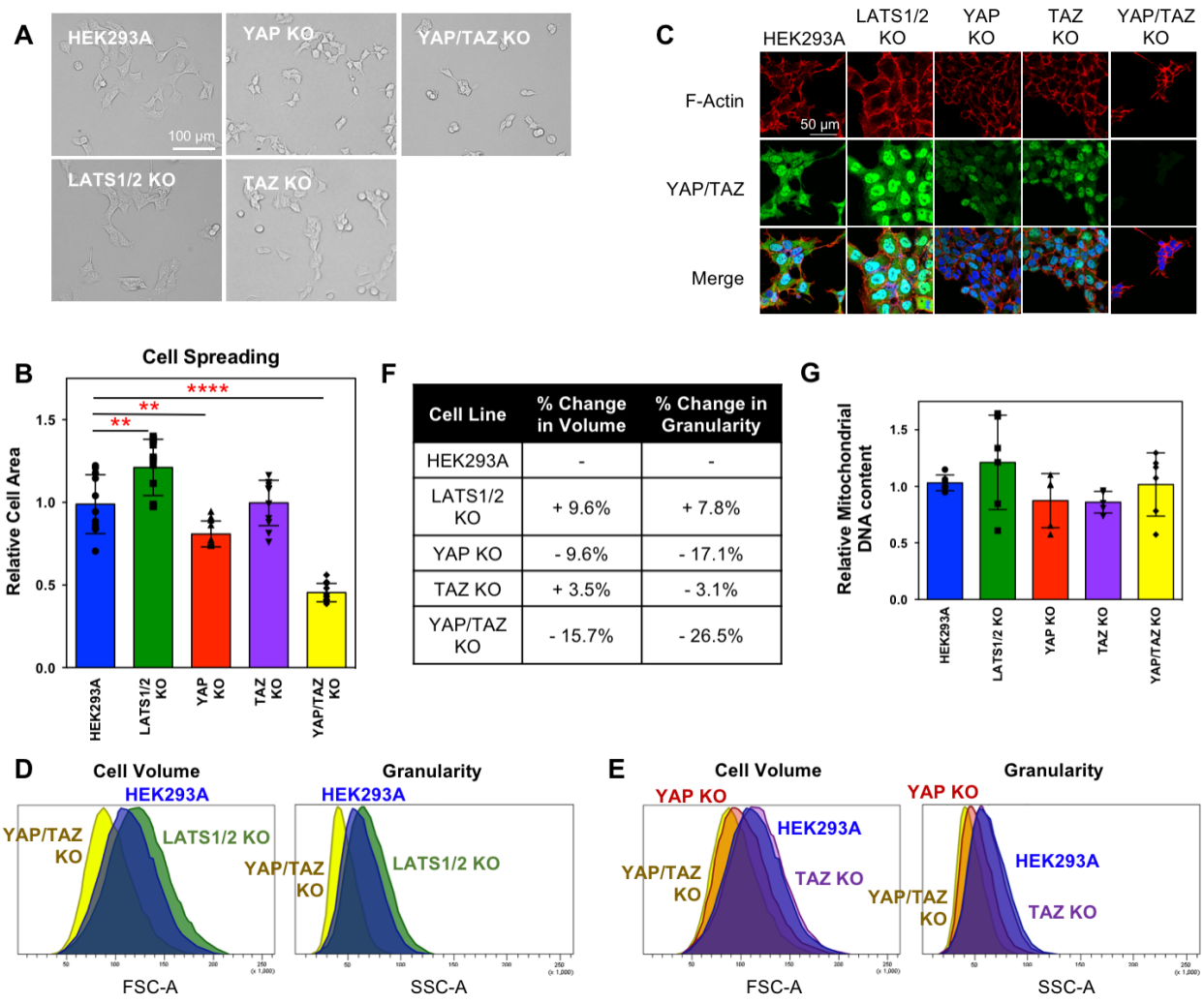


Figure 4.1. Comparison of YAP and TAZ knockout cells.
A. Schematic of the structural differences between YAP and TAZ. The domains are labeled as PRL (Proline-rich region), TBD (TEAD binding domain), WW (WW domain), SH3 (SH3 binding domain), TAD (Transcriptional activation domain), PXXOP (PxxOP site), 14-3-3 (14-3-3 binding site), CC (Coiled-coil region), PDZ (PDZ binding domain), or PD (Phospho-degron). B. Western blots showing loss of LATS1/2, YAP, and/or TAZ. Cells were serum starved overnight and either harvested or subsequently stimulated with serum for 60 minutes. Total cell lysates were used for Western blotting with the indicated antibodies. C. Immunofluorescence showing YAP/TAZ (red), TEAD1-4 (green), and DAPI (DNA stain, blue) localization following either overnight serum starvation or overnight serum starvation followed by 60 min serum stimulation. D-F. qPCR of CTGF, CYR61, and LGR5 following either overnight serum starvation (white bars) or overnight serum starvation followed by 60 min serum stimulation (grey bars). Data represented as +/- SD.



Cell Line	% Change in Volume	% Change in Granularity
HEK293A	-	-
LATS1/2 KO	+ 9.6%	+ 7.8%
YAP KO	- 9.6%	- 17.1%
TAZ KO	+ 3.5%	- 3.1%
YAP/TAZ KO	- 15.7%	- 26.5%

Figure 4.2. Loss of YAP results in decreased cell spreading, volume, and granularity. A. Bright-field images showing cell spreading on dish. B. Quantification from part A. Data represented as +/- SD. C. Immunofluorescence showing F-actin (red), YAP/TAZ (green), or DAPI (DNA stain, blue). D-E. FACS data showing differences in cell volume (FSC) and granularity (SSC) for the double knockout cells (D) and single knockout cells (E). F. Quantification from part D and E. G. PCR quantification of the relative mitochondrial DNA content for each cell line. Data represented as +/- SD.

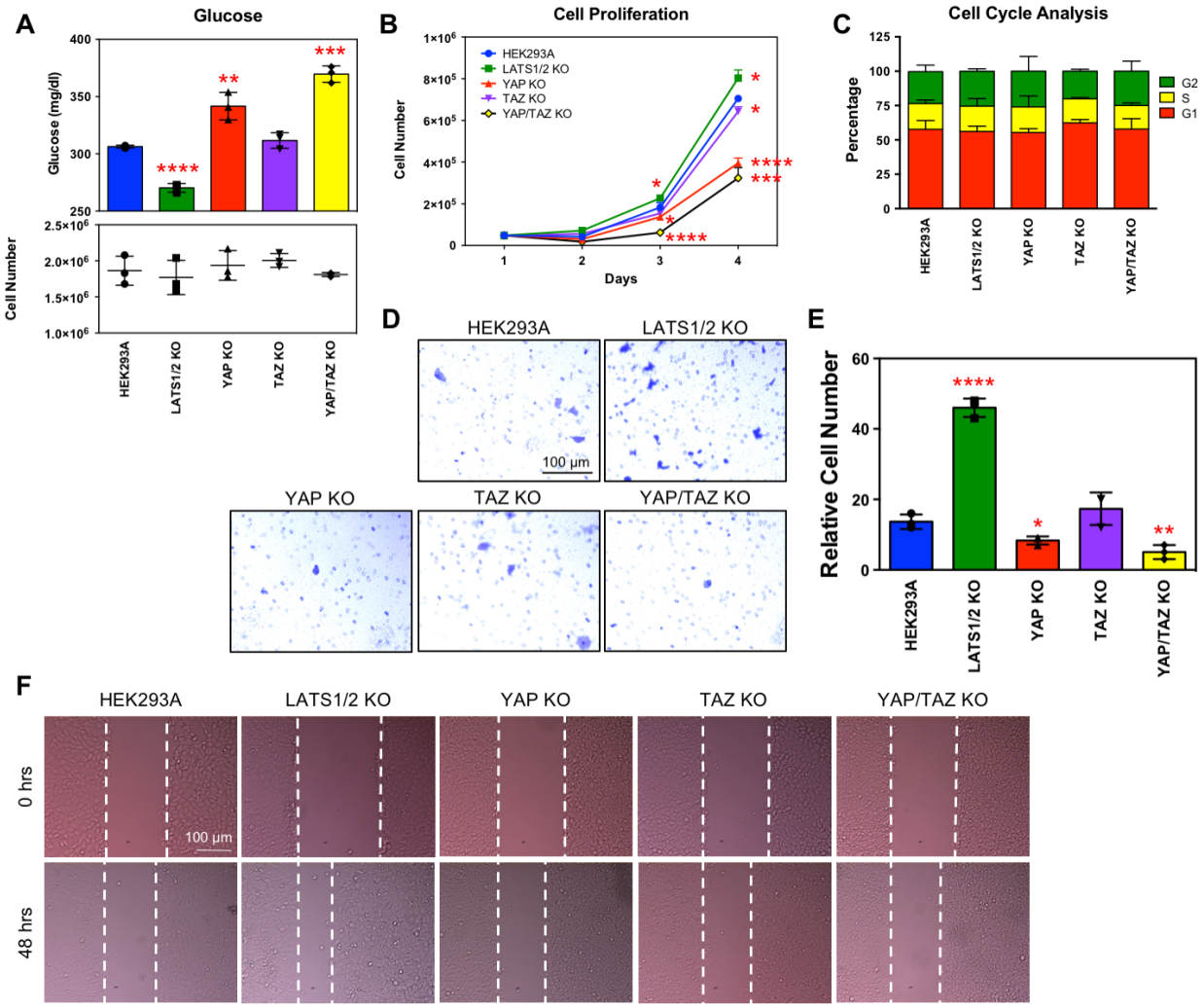


Figure 4.3. Loss of YAP results in decreased glucose uptake, proliferation, and migration.
A. Glucose levels remaining in the culture media following 6 hour incubation. Data represented as +/- SD. B. Cell proliferation curves for each of the cell lines under normal growth conditions. Data represented as +/- SD. C. Cell cycle analysis of each of the cell lines under normal growth conditions. Data represented as +/- SD. D. Bright-field images showing migration through a 8um PET pore membrane after 8 hours. E. Quantification from part D. Data represented as +/- SD. F. Bright-field images showing migration following a scratch assay.

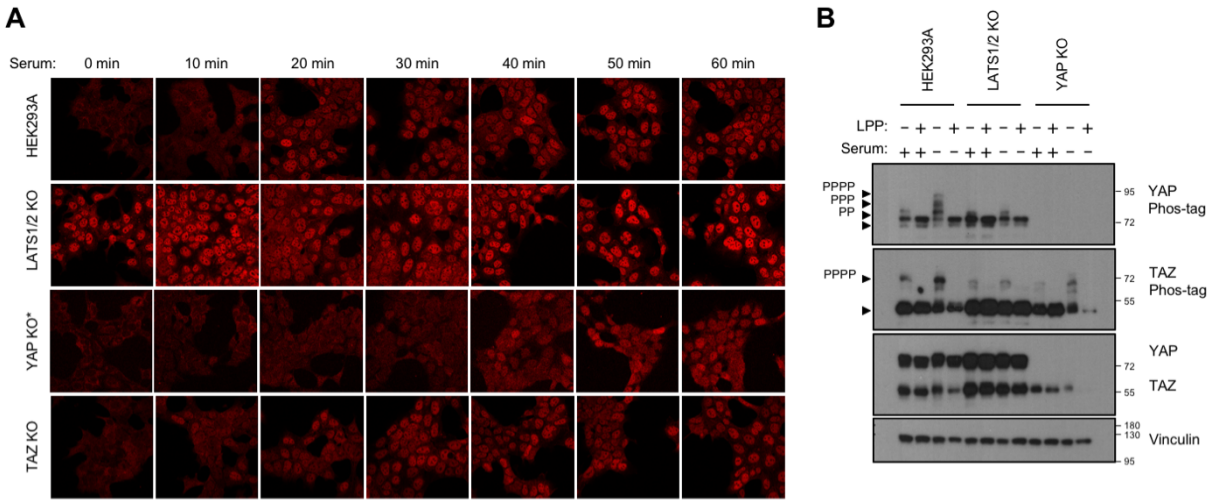


Figure 4.4. Comparing kinetics of YAP and TAZ activation.
A. Immunofluorescence showing a time-course of YAP/TAZ (red) localization in response to serum stimulation following overnight serum starvation. B. Western blots showing YAP and TAZ phosphorylation in response to serum starvation and lambda phosphatase treatment.

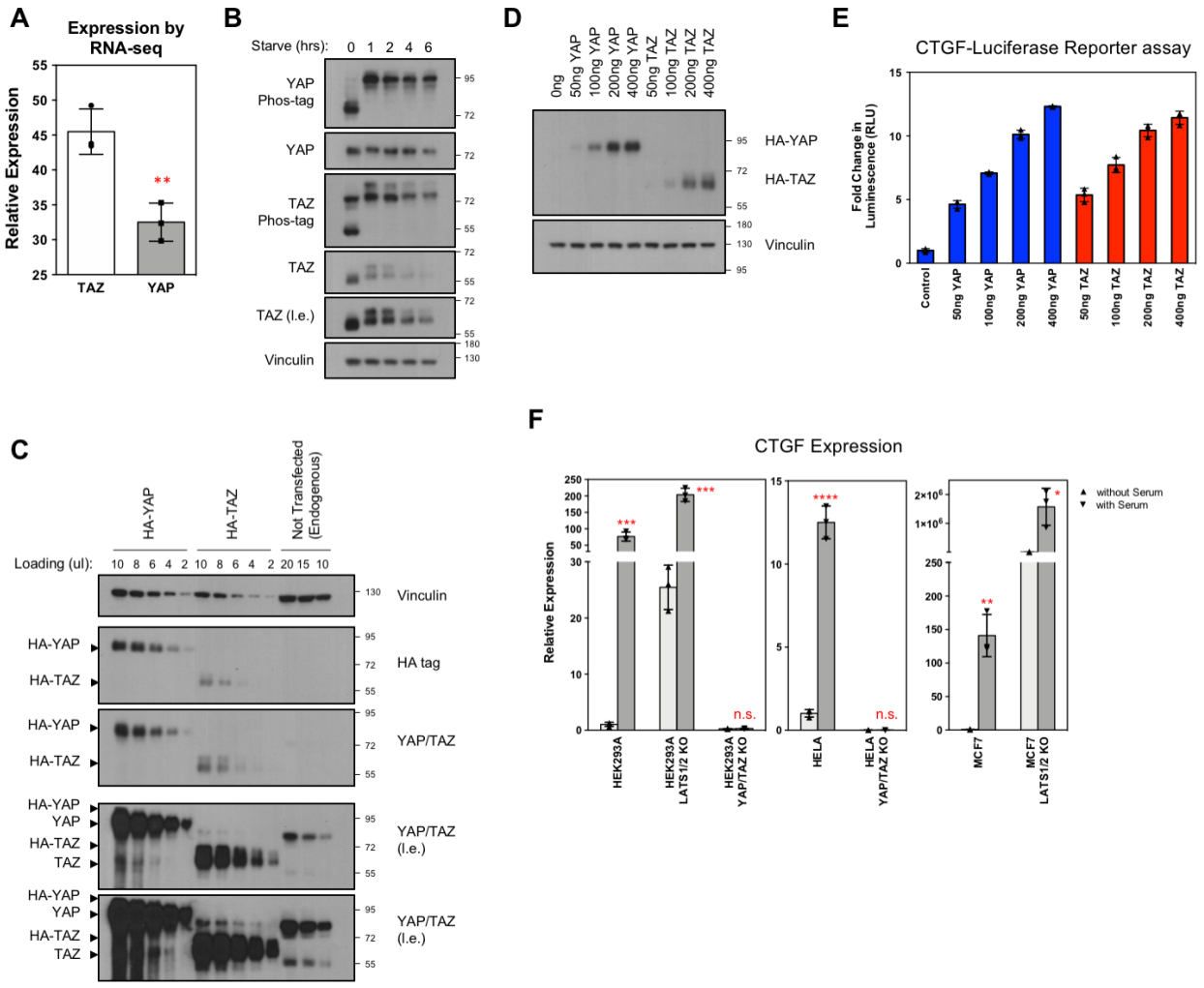


Figure 4.5. Comparing YAP and TAZ protein expression.
A. mRNA expression levels of TAZ and YAP as detected by RNA-seq. Data represented as +/- SD. B. Western blots showing changes in YAP and TAZ phosphorylation and protein levels following serum starvation for the indicated times. C. Western blots comparing detection of HA-YAP and HA-TAZ using either an HA antibody or the YAP/TAZ antibody, and detection of endogenous YAP and TAZ using the YAP/TAZ antibody. D. Western blots showing HA-YAP and HA-TAZ expression following transfection along with a CTGF-luciferase reporter in the YAP/TAZ KO cells. E. Luciferase activity following transfection of a CTGF-luciferase reporter with varying amounts of either HA-YAP or HA-TAZ in the YAP/TAZ KO cells. Data represented as mean +/- SD. F. qPCR of CTGF expression in various cell lines as indicated following either overnight starvation (white bars) or overnight starvation followed by serum stimulation for 90 minutes (grey bars). Data represented as mean +/- SD.

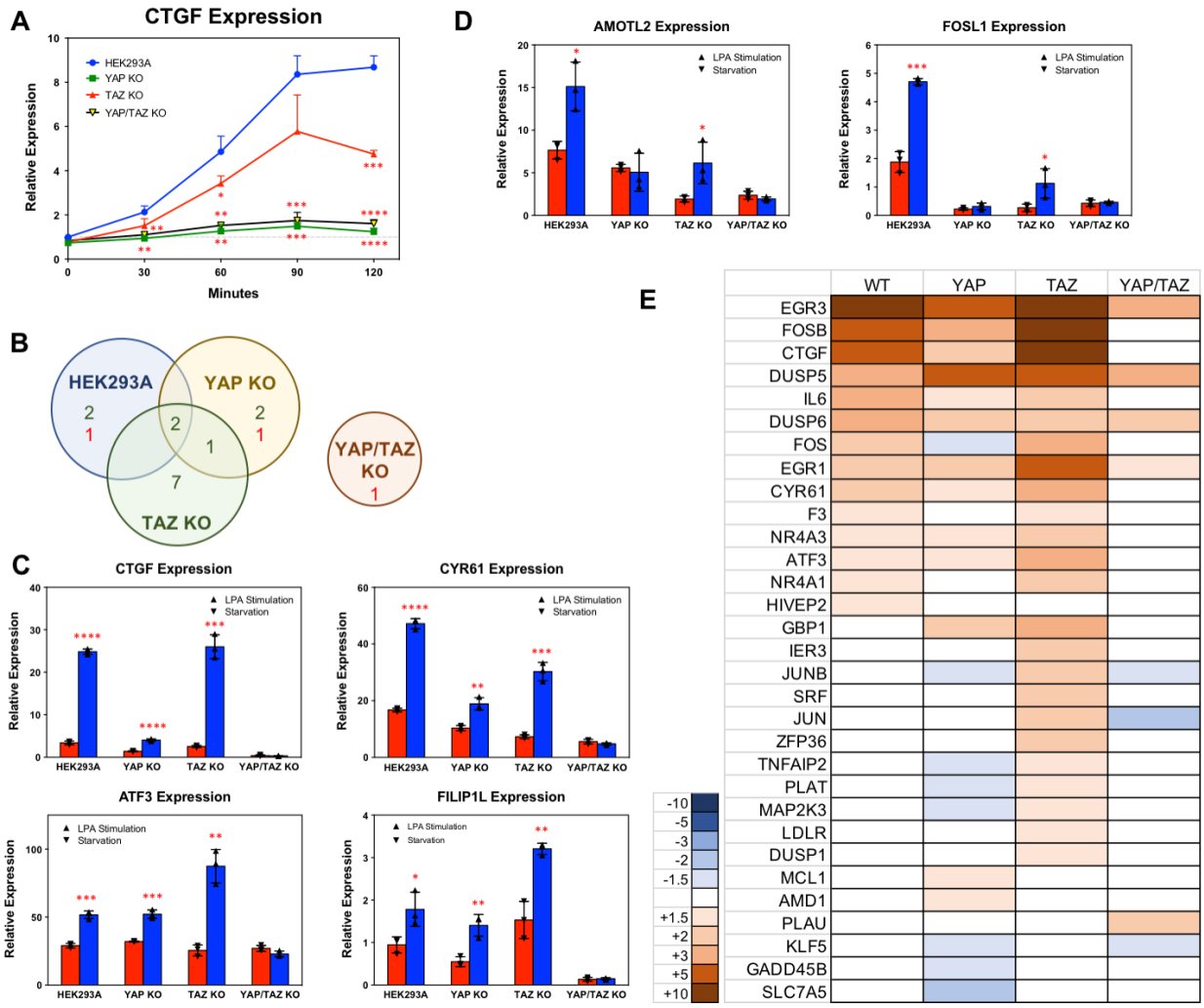


Figure 4.6. Using RNA-seq to examine transcriptional differences between YAP and TAZ.

A. qPCR time-course of CTGF expression following overnight serum starvation and treatment with LPA for the indicated times in the wild-type, YAP KO, TAZ KO, and YAP/TAZ KO cells. Data represented as +/- SD. B. Schematic representing genes which were statistically significant and showed more than a 2-fold change following LPA stimulation. Genes in green represent a 2-fold induction, and genes in red represent a 2-fold decrease in expression following stimulation. C. Relative expression from RNA-seq of genes which are YAP/TAZ-dependent following serum starvation (red bars) or LPA stimulation (blue bars). Data represented as +/- SD. D. Relative expression from RNA-seq of genes which are YAP-dependent following serum starvation (red bars) or LPA stimulation (blue bars). Data represented as +/- SD. E. Heat map summarizing induction of genes in the wild-type, YAP KO, TAZ KO, and YAP/TAZ KO cell lines in response to LPA stimulation by RNA-seq.

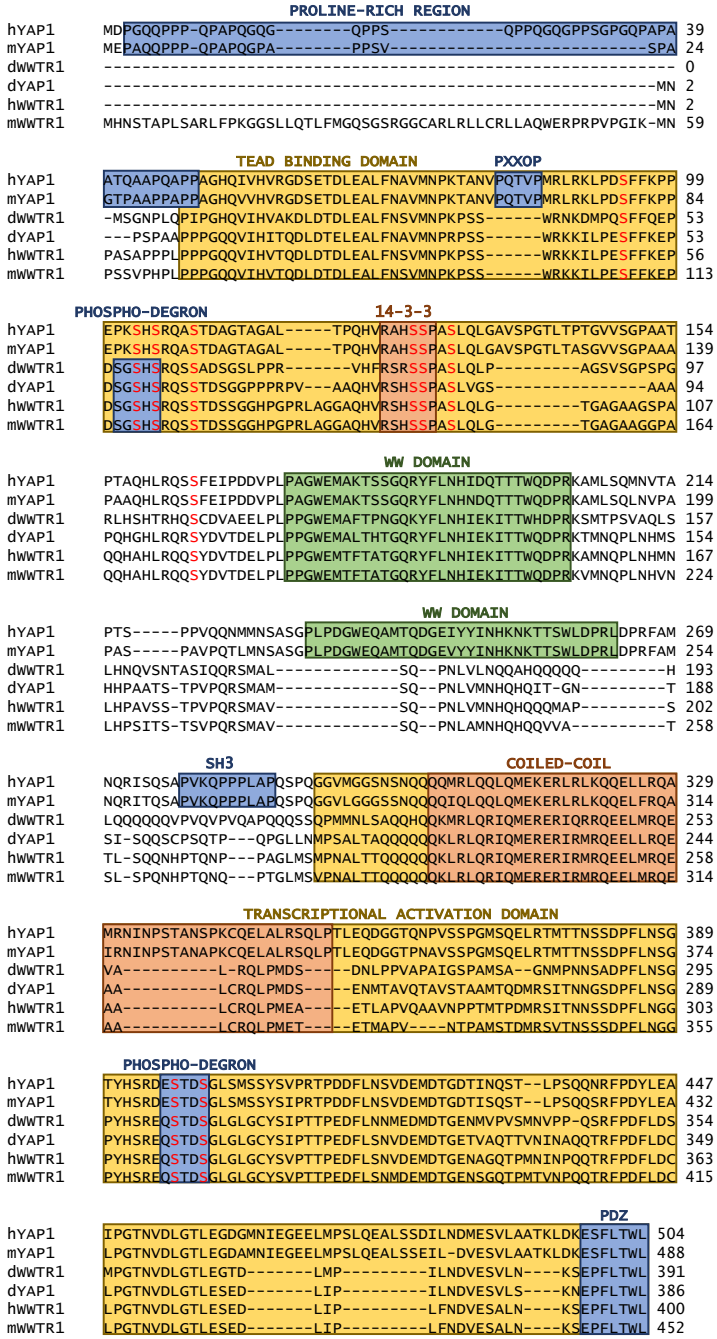


Figure S4.1. Protein alignment of YAP and TAZ.
Protein alignment of YAP1 and TAZ (WWTR1) in homo sapiens (h), mus musculus (m), and danio rerio (d). Conserved serines are highlighted in red. Protein domains are highlighted and labeled accordingly.

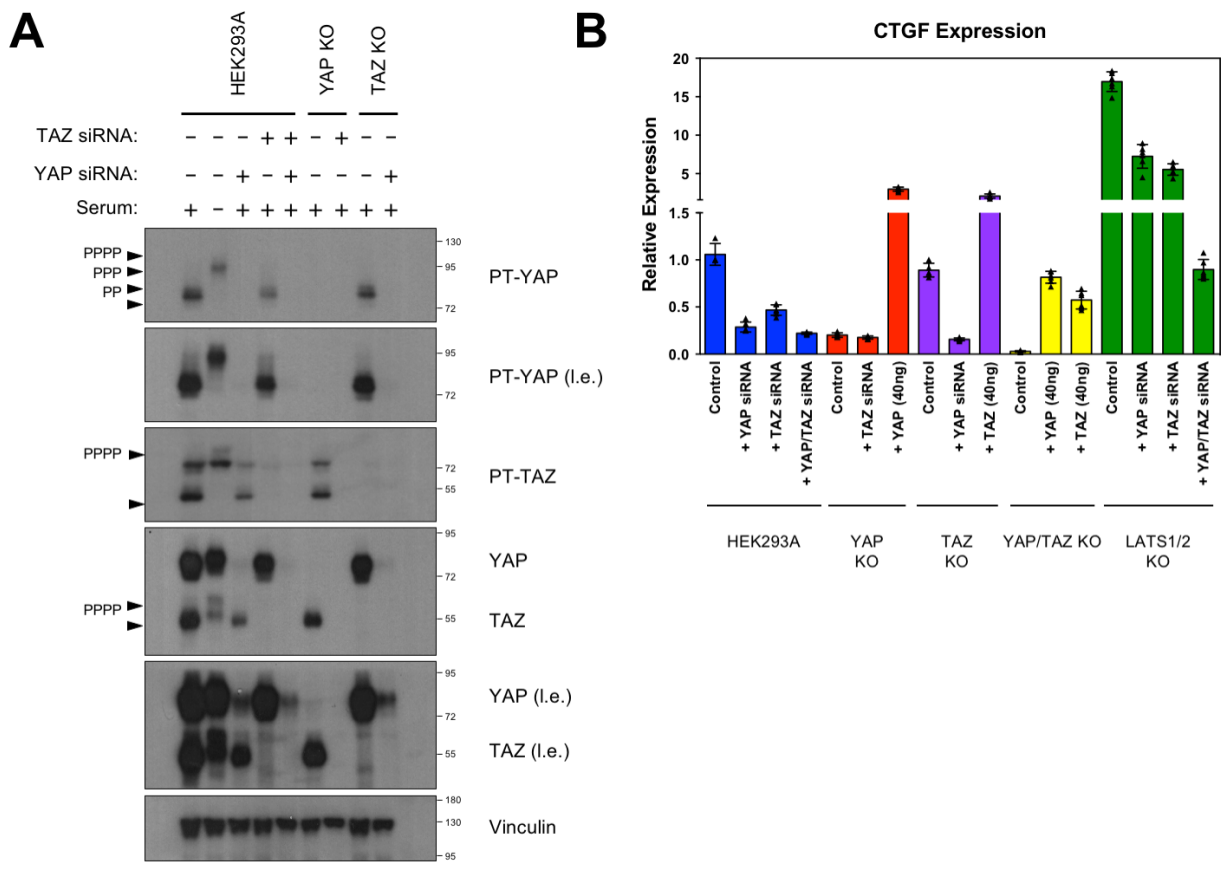


Figure S4.2. Rescue of YAP and TAZ knockout cells.
A. Western blots showing knockdown efficiency of YAP and TAZ siRNA. B. qPCR for CTGF expression following either YAP or TAZ siRNA knockdown or YAP/TAZ rescue in the YAP KO, TAZ KO, YAP/TAZ KO, and LATS1/2 KO cell lines. CTGF expression can be rescued in the knockout cells by transfection of wild-type YAP or TAZ. Data represented as +/- SD.

A

Construct	Mutation	Notes
1	S94A	Phosphorylation of S94 prevents TEAD binding
2	S103A / S105A	Phosphorylation of analogous resides in TAZ create a phospho-degron
3	S109A	
4	S127A	Phosphorylation of S127 is responsible for 14-3-3 binding
5	S128A	Phosphorylation of S128 interferes with 14-3-3 binding
6	S131A	
7	S163A / S164A	
8	WW1 deletion	WW domains mediate many protein-protein interactions responsible for YAP localization (e.g. LATS1/2, AMOT)
9	WW2 deletion	WW domains mediate many protein-protein interactions responsible for YAP localization (e.g. LATS1/2, AMOT)
10	WW1 / WW2 deletion	WW domains mediate many protein-protein interactions responsible for YAP localization (e.g. LATS1/2, AMOT)

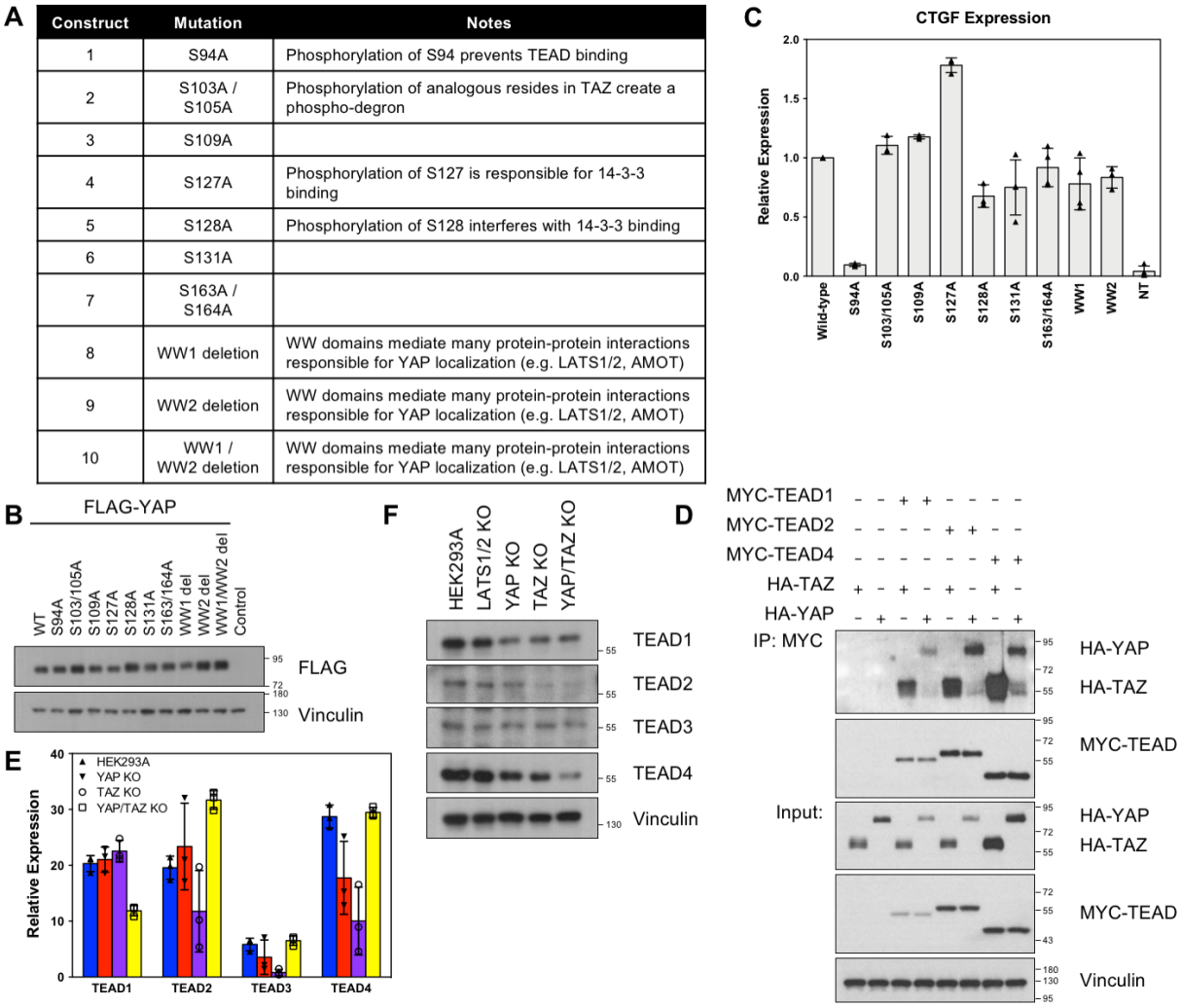


Figure S4.3. Characterizing YAP/TAZ interaction with TEAD.
A. Table indicating mutations made in either the conserved serine residues or WW domains of YAP. B. Western blots showing expression of the mutant YAP constructs in YAP/TAZ KO cells. C. qPCR of CTGF expression following expression of the mutant YAP constructs in YAP/TAZ KO cells. Cells were harvested under normal growth conditions. Data represented as +/- SD. D. Western blots showing co-immunoprecipitation of YAP and TAZ with TEAD1/2/4. E. RNA expression levels of TEAD1, TEAD2, TEAD3, and TEAD4 in the indicated knockout cells by RNA-seq. Data represented as +/- SD. F. Western blots showing expression of TEAD1, TEAD2, TEAD3, and TEAD4 in the various knockout cell lines.

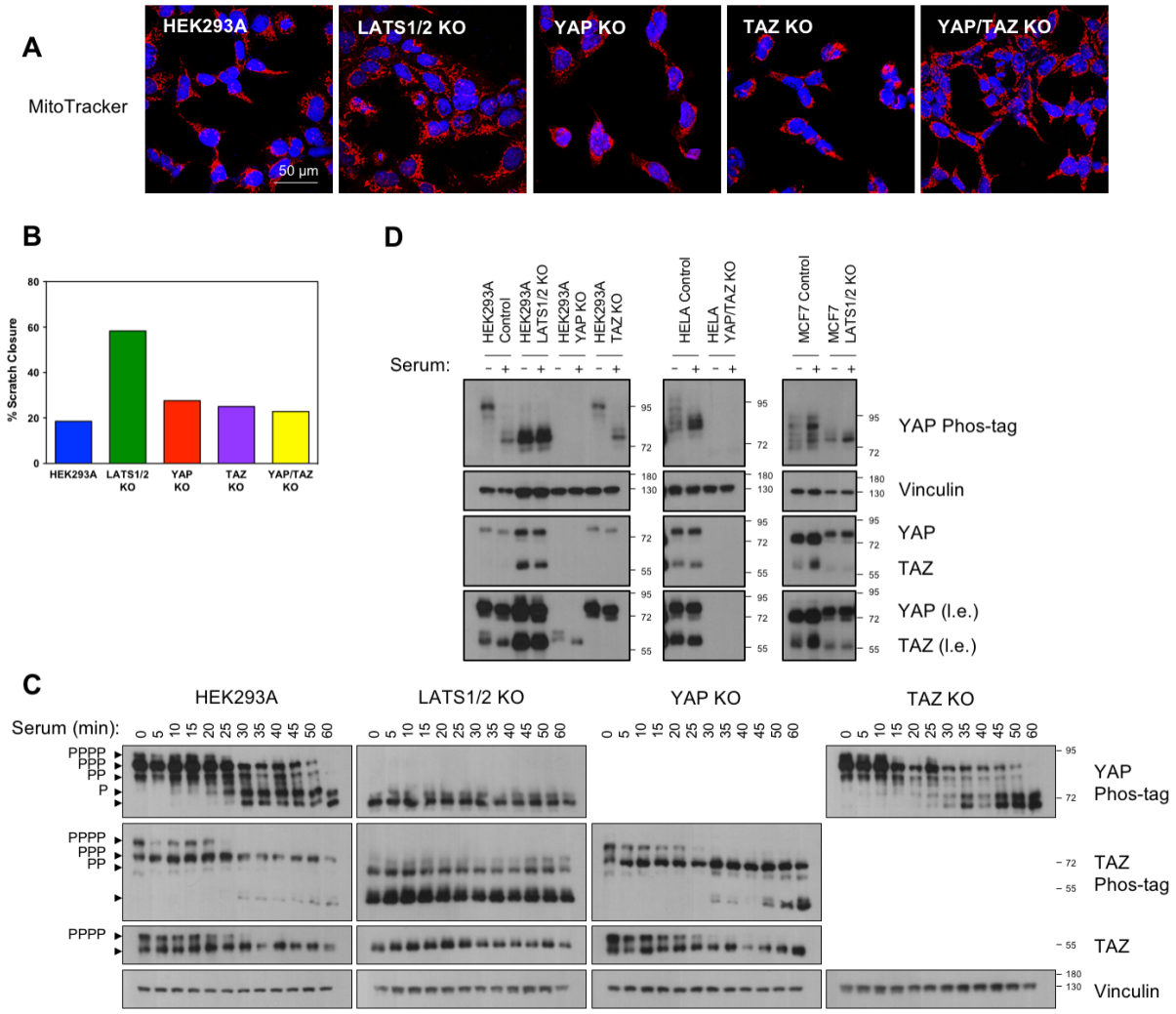


Figure S4.4. Effect of YAP/TAZ inactivation on mitochondria, migration, and kinetics of YAP/TAZ activation.
A. Immunofluorescence staining of the mitochondria with Mitotracker (red) and DAPI (DNA stain, blue). B. Quantification of the percent wound closure from the scratch assay in Figure 4.3F. C. Western blots showing phosphorylation status of various cell lines following overnight serum starvation or overnight serum starvation followed by serum stimulation for 90 minutes. D. Western blots showing YAP and TAZ phosphorylation status by phos-tag following serum stimulation after overnight serum starvation.

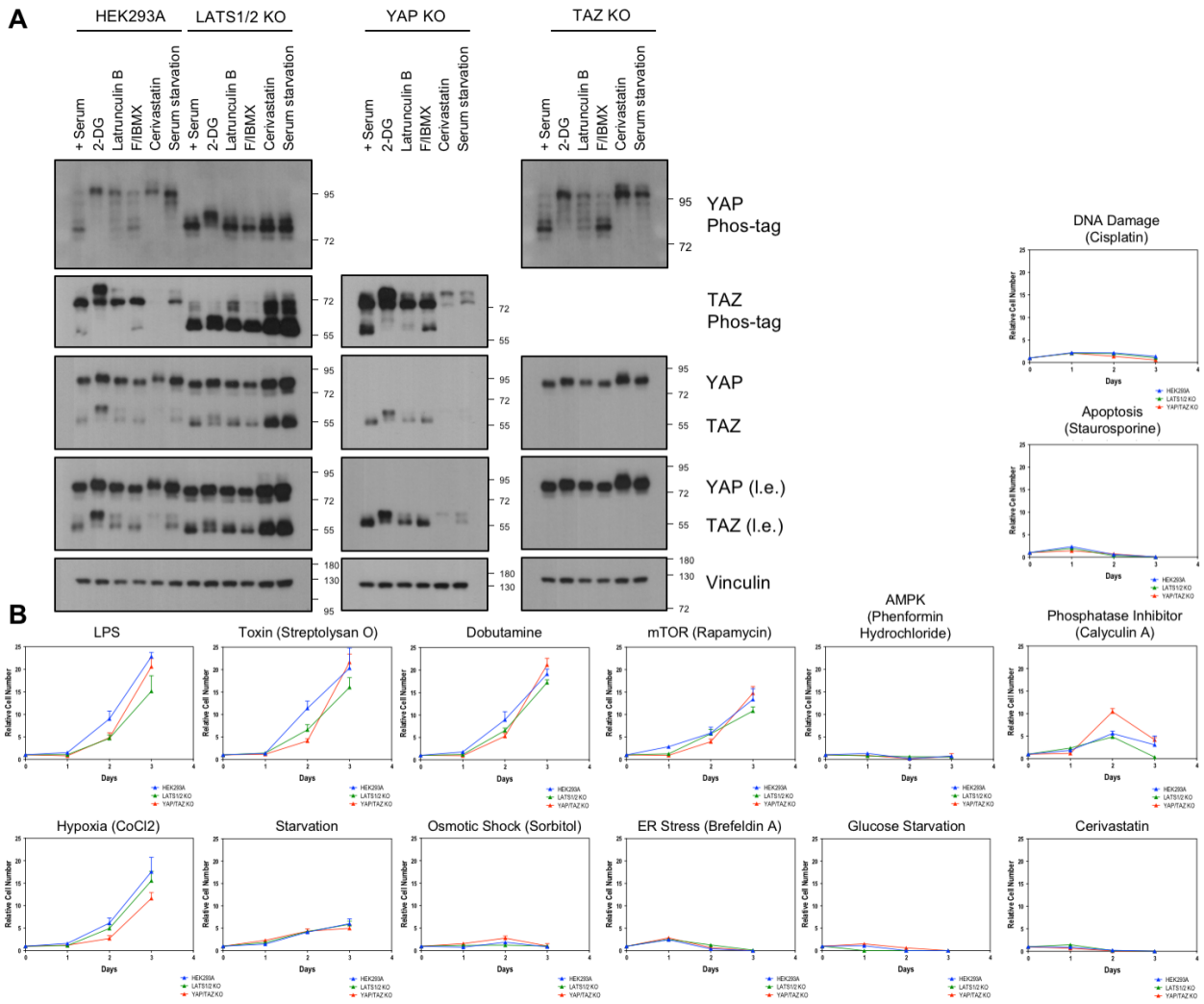


Figure S4.5. Analyzing effect of various drugs and cellular stresses on YAP/TAZ. A. Western blots showing YAP and TAZ phosphorylation status by phos-tag following treatment with the following drugs: 2-DG (2 mM 2-Deoxy-D-Glucose in glucose-free DMEM, 90 minutes), Latrunculin B (250 ng/ml, 75 minutes), F/IBMX (10 uM Forskolin, 100 uM 3-isobutyl-1-methylxanthine, 60 minutes), Cerivastatin (1 uM, overnight/18 hours), or serum starvation (overnight/18 hours). B. Cell proliferation and survival in the wild-type, LATS1/2 KO, and YAP/TAZ KO cells in response to different indicated stimuli. Data represented as +/- SD.

A

	% Genes
Molecular Function	
Nucleic acid binding (GO:0003676)	28
mRNA binding (GO:0003729)	3
Sequence-specific DNA binding transcription factor activity (GO:0003700)	25
Protein binding (GO:0005515)	38
Transcription factor binding transcription factor activity (GO:0000989)	6
Protein Class	
Transcription factor (PC00218)	25
Growth factor (PC00112)	6

B

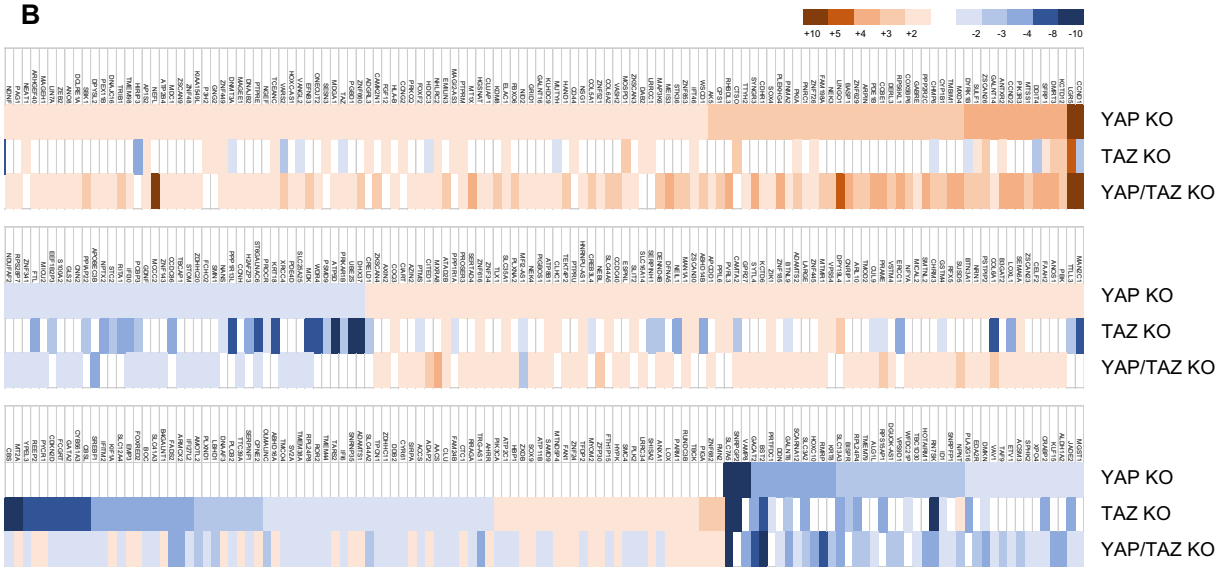


Figure S4.6. GO pathway analysis of RNA-seq data.
A. Summary of the GO pathway analysis of genes induced following LPA stimulation shown in Figure 4.6E. C. Heat map showing genes identified by RNA-seq with a 1.5-fold or greater difference in the YAP KO, TAZ KO, or YAP/TAZ KO cells relative to the wild-type cells under baseline starvation conditions.

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Chapter 5: Conclusion

5.1 Conclusion and future directions

The Hippo pathway is an important regulator of tissue homeostasis and organ size. The Hippo pathway consists of a kinase cascade of MST1/2 and LATS1/2, along with their adaptor proteins SAV1 and MOB1A/B. When activated, LATS1/2 phosphorylates the downstream effectors of the Hippo pathway, transcription co-activators YAP and TAZ. When YAP/TAZ are phosphorylated at S127, they are sequestered in the cytoplasm by 14-3-3, ubiquitinated, and degraded. Conversely, when the Hippo pathway is inactivated, dephosphorylated YAP/TAZ translocate to the nucleus where they interact with a host of transcription factors including TEAD1-4 to induce expression of genes promoting cell proliferation and cell growth.

Accordingly, dysregulation of YAP and TAZ has been associated with many human diseases and cancers (Plouffe et al., 2015). In many cases, increased YAP/TAZ expression and nuclear localization have been correlated with poor prognosis and an increased risk of metastasis. However, how the Hippo pathway and YAP/TAZ become dysregulated is not clear. Few mutations have been identified in either YAP, TAZ, or the core Hippo pathway kinases (Harvey et al., 2013). One possibility is that mutations or changes in expression or activity of upstream regulators of the Hippo pathway may contribute to aberrant YAP/TAZ activity during pathogenesis.

YAP/TAZ regulation of desmosomes

The oncogenic role of YAP and TAZ has been well-documented. However, the interplay between how YAP/TAZ regulate intercellular junctions, as well as how these junctions may in turn regulate YAP/TAZ activity has not been well explored. Active YAP/TAZ has been associated with cells undergoing an epithelial-to-mesenchymal (EMT) transition and attaining cancer stem cell-like properties, including the loss of intercellular junctions and gaining the ability to migrate (Diepenbruck et al., 2014; Lei et al., 2008; Liu et al., 2010). On the other hand, desmosomes are large intercellular junctions which are responsible for maintaining cell-cell adhesion to resist mechanical stress. Although traditionally viewed as being relatively static entities, recent work has identified several core components of desmosomes as being capable of modulating intracellular signaling including Wnt signaling (Green and Simpson, 2007; Yang et al., 2012). Furthermore, down-regulation of several desmosome components has been correlated with cancer progression and increased risk of metastasis, and some are even used as diagnostic markers (Boelens et al., 2007; Cui et al., 2012; Papagerakis et al., 2009). However, the mechanisms by which their expression is regulated and how they may regulate intracellular signaling is not clear.

We found that over-expressing YAP or activating endogenous YAP by serum or LPA stimulation was sufficient to repress mRNA expression of desmosome components (Figure 2.1B-G). Expression of desmosome components Desmocollin 2 (DSC2), Plakoglobin (JUP), and Desmoplakin (DSP) is actually fairly dynamic, and significant differences in protein levels are evident after only 4 hours of treatment with the translation inhibitor cycloheximide (Figure 2.2E). Thus, YAP/TAZ transcriptional repression of these components is sufficient to result in fairly dramatic changes in

protein expression as well (Figure 2.2B). This repression is mediated by ZEB1/2, which are induced by YAP/TAZ in a TEAD1-4-dependent manner. When comparing RNA microarray data from the Cancer cell line encyclopedia (CCLE), there are significant negative correlations between ZEB1 expression and expression of these desmosome components, suggesting that this mechanism may be conserved in cancer progression (Figure 2.3D). Finally, to determine whether changes in expression of desmosome components can feed back to regulate YAP/TAZ signaling, we used shRNA to knockdown expression of the obligatory desmosome component DSP in HaCaT human keratinocytes. Not only was DSP knockdown sufficient to induce expression of YAP/TAZ target genes CTGF and CYR61, but the knockdown cells also migrated significantly faster than the control cells in a scratch assay (Figure 2.4E). This raises the possibility that loss of desmosomes and perhaps other intercellular junctions are not only regulated by YAP/TAZ activity through transcriptional repression, but that they can also feed back to regulate the Hippo pathway as well. Although aberrant YAP/TAZ activity has been correlated with many human cancers, how YAP/TAZ become dysregulated is not clear. Therefore, if other mutations or signaling pathways become corrupted to induce EMT, the loss of desmosomes may actually feed back to activate YAP and TAZ to promote further migration, proliferation, and cancer progression.

Characterizing Hippo pathway components through gene inactivation

The Hippo interactome is quite extensive. Several Mass Spectrometry-based studies have together identified hundreds of proteins which directly interact with the core Hippo pathway kinases and may affect their localization, phosphorylation, and

activity (Couzens et al., 2013; Kwon et al., 2013; Wang et al., 2014). Thus, it is necessary to identify which components are most critical for regulating YAP/TAZ, because this may provide clues as to how YAP/TAZ become dysregulated. While some of these components have already been reported, such as the Mitogen-Activated Protein Kinase kinase kinase kinase (MAP4K) family, AMPK (PRKKA1/PRKKA2), Ras Homology Family Member A (RHOA), Neurofibromin 2 (NF2), and Angiomotin (AMOT) , most studies only focus on the component of interest to that particular study. Thus, it is difficult to compare the relative contribution of each component because the cell types and culture conditions vary across studies.

To provide some clarity as to which components are the most physiologically relevant in regulating YAP/TAZ, we used CRISPR/Cas9 to create knockout cell lines for several of these components and compared their relative contributions in regulating YAP/TAZ in response to several conditions. Our goal was to provide greater insight into the Hippo pathway interactome and, if we identified components which have a strong regulatory effect on Hippo pathway activity, to determine whether we could identify any correlation between mutations in those components and dysregulated YAP/TAZ in disease.

First, we conclusively demonstrated that LATS1/2 are the primary direct kinases for YAP/TAZ (Figure 3.2A). When LATS1/2 are inactivated, YAP/TAZ are strongly dysregulated in response to all conditions tested. This also corroborates with reported LATS1/2 conditional KO animal models; inactivation of LATS1/2 in the liver results in massive hepatomegaly that is YAP/TAZ-dependent (Chen et al., 2015). Additionally, inactivating mutations or promoter methylation of LATS1/2 has been associated with

increased YAP/TAZ expression in several cancers, including renal cell carcinoma and lung and colorectal cancers, suggesting that LATS1/2 regulation of YAP/TAZ is relevant in multiple tissue and cell types and has clear physiological and pathological implications (Cao et al., 2014; Chen et al., 2014; Lin et al., 2014; Wierzbicki et al., 2013).

Furthermore, although LATS1/2 have some low levels of intrinsic kinase activity towards YAP/TAZ, MOB1A/B are required for full phosphorylation and activation of LATS1/2 (Figure 3.5E). Interestingly, neither MOB1A/B (T35) or LATS1/2 (HM) phosphorylation were sufficient to predict LATS1/2 kinase activity towards YAP/TAZ, as YAP/TAZ were strongly phosphorylated in the MST1/2 KO cells even in the absence of MOB1A/B (T35) phosphorylation, and YAP/TAZ were clearly dephosphorylated even in the presence of phosphorylated LATS1/2 (HM) in the NF2 KO cells (Figure 3.5E). Even though MST1/2, the MAP4K family, and TAOK1/3 can directly phosphorylate LATS1/2 (HM), the current model is that MOB1A/B are still required for auto-phosphorylation of the LATS1/2 activation loop (Chan et al., 2005; Praskova et al., 2008). These observations raise the possibility that the Hippo pathway may be therapeutically manipulated by targeting either MOB1A/B or LATS1/2, since both MOB1A/B and LATS1/2 are required to phosphorylate and inactivate YAP/TAZ.

Second, as previously mentioned, inactivating NF2 alone was sufficient to significantly disrupt YAP/TAZ phosphorylation (Figure 3.2D). This was independent of LATS1/2 protein stability or even phosphorylation of its hydrophobic motif. NF2 does not affect upstream MST1/2 kinase activity because phosphorylated MOB1A/B (T35) is clearly present and unaffected by NF2 deletion. NF2 recruits LATS1/2 to the plasma

membrane, where it is phosphorylated by MST1/2; thus, it is possible that deletion of NF2 affects LATS1/2 localization (Yin et al., 2013). NF2 is one of the few known instances where a mutation in a Hippo pathway component has direct disease implications; in Neurofibromatosis type 2, loss of function mutations in NF2 and subsequent increased YAP expression results in the development of schwannomas and meningiomas (Schulz et al., 2014; Striedinger et al., 2008). However, the observation that YAP/TAZ can still become phosphorylated in response to strong stimuli such as high cell density or glucose starvation raises the possibility that, under certain conditions, LATS1/2 can still become localized at the plasma membrane and interact with MST1/2 and MOB1A/B in an NF2-independent manner, become activated, and that YAP/TAZ can still be therapeutically inhibited in NF2-mutant patients.

Third, we demonstrated that, in addition to what has been previously published about TAOK1/3 acting upstream of the Hippo pathway to phosphorylate and activate MST1/2, TAOK1/3 can also directly phosphorylate LATS1/2 independent of MST1/2 (Figure 3.6B). Although it is clear both from what has been previously published and from our own results that MST1/2 and the MAP4K family are the primary activators of LATS1/2, the finding that TAOK1/3 are also direct kinases for LATS1/2 highlights how tightly regulated LATS1/2 and YAP/TAZ are (Li et al., 2014; Meng et al., 2015; Zheng et al., 2015). Deleting TAOK1/3 had a significant effect on YAP/TAZ phosphorylation in response to cell-cell contact; deleting all three kinase families (MST1/2, the MAP4K family, and TAOK1-3) almost completely abolished YAP/TAZ phosphorylation (Figure 3.4B). It is possible that each of these kinase families are activated in response to distinct types of stimuli or cellular stress, or that their regulation of LATS1/2 is not

necessarily a one-to-one ratio of stimuli to kinase family but more of an additive effect, where the level of LATS1/2 activation is dependent on how many of these kinase families are activated. Regardless, this finding adds a new level of complexity in Hippo pathway regulation.

Finally, we found that RHOA plays a critical role in mediating growth signals from GPCRs to regulate YAP/TAZ. In the RHOA KO cells, even serum was unable to inactivate LATS1/2 and induce YAP/TAZ dephosphorylation (Figure 3.2D). Although RHOA and regulation of the actin cytoskeleton have long been thought to play a role in mediating signals from GPCRs to the Hippo pathway, to our knowledge this is the first genetic data supporting a clear role for RHOA. This finding is significant because it demonstrates how activating or inactivating mutations in upstream components can significantly disrupt Hippo pathway signaling and contribute to dysregulated YAP/TAZ signaling in disease, as well as how upstream components can be targeted to negatively regulate YAP/TAZ activity.

Therefore, our study provides a useful resource and greater clarity as to which components of the Hippo pathway are most critical for regulating YAP/TAZ, the downstream effectors of the Hippo pathway. Besides what is already well-established for NF2 mutations in Neurofibromatosis type 2, we did not identify any additional correlations between mutations, amplifications, or deletions of either RHOA or TAOK1/3 and dysregulated YAP/TAZ in human disease. Further work is needed to identify novel upstream components which may contribute to dysregulated YAP/TAZ; identifying and understanding these relationships will be critical to grasp how the Hippo pathway becomes disrupted and for identifying potential therapeutic targets. Intriguing

possibilities include utilizing genome-wide knockout and activating CRISPR/Cas9 libraries, which will enable us to identify upstream components which affect the Hippo pathway and YAP/TAZ activity without necessarily any direct interaction (Konermann et al., 2015; Shalem et al., 2014). Another possibility is that, due to the complex nature of regulation of the Hippo pathway, that dysregulated YAP/TAZ in disease is due to mutations in any number of other signaling pathways, and that dysregulation of YAP/TAZ is important for cancer progression but itself is not one of the primary drivers of cancer. However, regardless of whether this is the case, it will still be important to target the Hippo pathway because it has been shown to play such a significant role in cancer progression.

Physiological consequences of dysregulated YAP and TAZ

YAP and TAZ are generally thought to be functionally redundant, although there are evolutionary, structural, and physiological clues which suggest they may have non-overlapping roles. For instance, while orthologs of YAP first appear in single cell eukaryotes, TAZ only appears in vertebrates (Hong et al., 2005; Sebe-Pedros et al., 2012). Structurally, while they share a high protein sequence similarity, YAP contains several additional domains not found in TAZ, while TAZ has an additional phosphodegron which causes its protein stability to be much more dynamically regulated than that of YAP (Huang et al., 2012). Finally, there are physiological differences between YAP and TAZ. YAP knockout mice are embryonic lethal, while TAZ knockout mice are partially lethal, although those surviving develop renal cysts and lung emphysema (Hossain et al., 2007; Makita et al., 2008; Morin-Kensicki et al., 2006; Tian

et al., 2007). This indicates that YAP and TAZ are not completely redundant because TAZ is unable to compensate for the loss of YAP, although this could be due to any number of reasons such as actual transcriptional differences or differences in tissue distribution.

In addition to better characterizing the upstream regulators of the Hippo pathway, we also wanted to better characterize the physiological consequences of dysregulated Hippo signaling. To this end, we compared LATS1/2 KO cells in which YAP/TAZ are constitutively-active, YAP/TAZ KO cells in which YAP/TAZ are constitutively-inactive, and YAP and TAZ single knockout cell lines. Our goal was to better understand the cellular effects of dysregulated YAP/TAZ signaling and determine whether there are any physiological or transcriptional differences between YAP and TAZ.

First, it is clear that dysregulation of YAP and TAZ has dramatic consequences on the cell. While inactivation of YAP in the YAP KO and YAP/TAZ KO cells had a significant negative effect on several cellular measures, including cell spreading, cell volume and granularity, glucose uptake and metabolism, cell proliferation, and migration, constitutive-activation of YAP/TAZ in the LATS1/2 KO cells had the opposite effect. The fact that so many of these phenotypes are particularly relevant in tissue overgrowth and cancer again raises the necessity of being able to target the Hippo pathway therapeutically. While inactivating TAZ also clearly had an effect on cellular physiology, as evidenced by the difference between the YAP KO and YAP/TAZ KO cells, overall the effect of deleting TAZ was not as significant as deleting YAP. We believe this is largely due to differences in protein expression; because TAZ protein stability is much more dynamically regulated, even though TAZ mRNA levels are

actually higher, YAP protein levels are significantly higher and more stable than that of TAZ (Figure 4.4E). When we co-transfected equal levels of YAP and TAZ, they were able to induce a luciferase reporter equally (Figure 4.4F-G). Thus, some of the physiological differences we observed may be due to differences in protein expression of YAP and TAZ. This may also explain some of the differences observed in the knockout mice; the relative levels of YAP and TAZ protein expression may easily be tissue or cell-type dependent.

Surprisingly, one difference we observed was in how YAP and TAZ phosphorylation is regulated. Following serum starvation, YAP becomes completely phosphorylated and inactive, while serum stimulation causes YAP to become completely dephosphorylated and nuclear (Figure S4.5A). Changes in phosphorylation status can be observed by phos-tag gel analysis. Likewise, in the absence of serum, TAZ is completely phosphorylated and inactive, and following prolonged starvation TAZ protein is degraded. However, following serum stimulation, TAZ does not become completely dephosphorylated (Figure S4.5A). This remaining phosphorylation is LATS1/2-independent, because it is present even in the LATS1/2 KO cells. While it is not clear whether there are any conditions in which TAZ becomes completely dephosphorylated, and it is not clear whether this phosphorylation has any physiological consequences in regulating TAZ protein stability, localization, or transcriptional activity, this raises the possibility that there may be conditions in which TAZ regulation is not completely identical to that of YAP.

Finally, when we performed RNA-seq to analyze differences between the YAP KO and TAZ KO cells, it is clear that there are differences between the cell lines.

However, some of this may be explained by the experimental design in which the cells were serum starved overnight before being stimulated with LPA. Because TAZ protein levels are dynamically regulated in response to starvation, following a prolonged overnight starvation, TAZ protein levels were probably already fairly low by the time of stimulation and collection. Additionally, the stimulus used was intentionally weak to isolate and identify direct targets of YAP/TAZ and hopefully minimize secondary or other further downstream effects. Nevertheless, even with these caveats, it is clear that YAP and TAZ are not completely redundant. For instance, there were genes such as AMOTL2 and FOSL1 whose induction was YAP-dependent but not TAZ-dependent. While clearly more work needs to be done, including varying the potency, duration, and type of stimuli, this raises the possibility that YAP and TAZ, although they are more similar than different, do have some differences in the transcriptional profiles they induce. Thus, this work provides a systematic functional analysis of YAP and TAZ and yields greater understanding into the cellular consequences of dysregulated YAP/TAZ signaling and some of the mechanisms by which YAP/TAZ may promote cancer progression.

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